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Antiplatelet medications and their indications in preventing and treating coronary thrombosis

Eric Van De Graaff and Steven R Steinhubl

Platelets play a pivotal role in the pathophysiology of unstable angina, acute myocardial infarction, and complications following percutaneous coronary intervention. Three classes of platelet-inhibiting drugs, aspirin, thienopyridines and platelet glycoprotein IIb/IIIa inhibitors, are now commonly used for the prevention and treatment of disorders of coronary artery thrombosis. For the last several decades aspirin has been the sole option for antiplatelet therapy in the treatment and prevention of the manifestations of cardiovascular disease. However, a wider selection of antiplatelet agents, including the thienopyridines (ticlopidine and clopidogrel) and the platelet glycoprotein (GP)IIb/IIIa receptor antagonists, are now available and provide clinicians with the opportunity to potentially improve upon the previous gold standard of aspirin. This review summarizes these drugs and the scientific data that have led to their use in primary and secondary prevention, unstable angina, myocardial infarction, and percutaneous coronary intervention.

Key words: acute coronary syndromes; aspirin; blood platelets; coronary disease; coronary thrombosis; myocardial ischaemia; platelet aggregation; platelet aggregation inhibitors; platelet glycoprotein GPIIb-IIIa complex.

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Introduction

Platelets are a principle component of arterial thrombus and therefore play a central role in the pathogenesis of acute myocardial infarction and thrombotic unstable angina. The mortality of these diseases combined with that of other thrombotic disorders, such as ischaemic stroke and pulmonary embolism, easily makes the platelet and its role in inappropriate thrombus formation a pivotal constituent of the deadliest pathological process in the developed world. With the recognition of the antiplatelet effects of aspirin in 1959 (1) and its reported benefit in the treatment of coronary thrombosis (2, 3), physicians began to target the platelet in their efforts to thwart the formation of unwanted clot. Over the years, other antiplatelet agents have come into use, with varying degrees of success, in the treatment and prevention of disorders of arterial thrombus formation.

The increasing array of antiplatelet drugs and their indications can be confusing, especially as this realm of therapy continues to develop at a rapid pace. This review will attempt to summarize the general function of three groups of antiplatelet medications, aspirin, the thienopyridines and the platelet glycoprotein (GP) IIb/IIIa receptor antagonists, and outline their specific indications in the field of cardiovascular medicine.

Platelet function and thrombosis in acute coronary syndromes

In 1912, Herrick first proposed that occlusion of a coronary artery is the underlying cause of myocardial infarction (4). Only decades later did the concept become generally accepted that abrupt vessel occlusion

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occurs as a result of thrombus formation (5, 6). In the last few years, we have gained great understanding of the interplay between atherosclerotic plaque and thrombus formation.

Characteristically, a lipid-rich arterial plaque fissures under the stress of haemodynamic and humoural triggers, thereby exposing the underlying matrix of thrombogenic substances to the circulating platelets (7, 8). Platelets are anuclear cell fragments shed by megakaryocytes in the bone marrow, and their function is to prohibit haemorrhage by coalescing into a thrombus at the site of damaged vascular endothelium.

When the integrity of the endothelium is disrupted, circulating platelets are exposed to collagen, von Willebrand factor (vWF) and fibrinogen in the subendothelial matrix. Specific glycoprotein receptors GP Ib and GP Ia-IIa on the surface of platelets actively bind vWF and collagen, respectively, and secure the platelets to the damaged endothelium. This binding, together with exposure to chemical mediators, such as collagen, epinephrine, adenosine diphosphate (ADP), thrombin and thromboxane, activates the platelets and causes them to degranulate and saturate the surrounding environment with chemotactins, clotting factors and vasoconstrictors.

Platelets bind to each other via GP IIb/IIIa receptors that are found in large numbers on the surface of platelets (9). Platelet activation leads to a conformational change in the GP IIb/IIIa receptor that allows binding of fibrinogen and vWF. As both of these proteins contain multiple binding sites for the GP IIb/IIIa receptor, they are able to act as a bridge by which activated platelets bind to each other (10) (Fig 1).

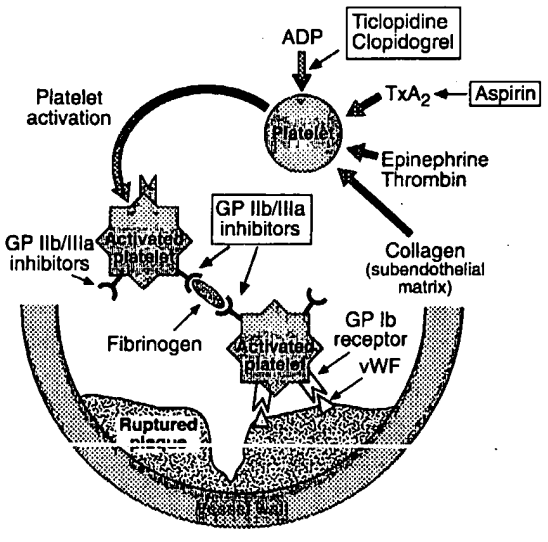

Figure 1. Location of action of principal antiplatelet drugs. ADP, adenosine diphosphate; GP, glycoprotein; TxA_2, thromboxane A_2; vWF, von Willebrand's factor.

As platelets coalesce and platelet activation continues, more GP IIb/IIIa receptors are expressed on the cell surface and become available for binding. Simultaneously, the activated platelet surface serves to accelerate the coagulation cascade and increase fibrin production. Ultimately, the burden of thrombus overwhelms the luminal area of the vessel, and blood flow ceases, potentially resulting in myocardial ischaemia and necrosis.

Therapy for preventing abnormal thrombus formation in the coronary circulation was initially aimed at the coagulation cascade (11), but, as the role of platelets in arterial thrombus became clearer, more clinicians focused their therapy on inhibition of platelet function and were met with surprising success (12). It is now clear that platelet inhibition is a cornerstone in the prevention and treatment of acute coronary syndromes.

Antiplatelet medications

Aspirin

While the analgesic and antipyretic effects of aspirin have been known for hundreds of years (13), only in the last half-century has it been established that aspirin inhibits platelet aggregation through irreversible acetylation and inactivation of the enzyme cyclooxygenase (COX) (14). The metabolite of COX, thromboxane A_2 (TxA_2), is a potent stimulus of vasoconstriction and platelet aggregation. A single dose of as little as 100 mg of aspirin will block all production of TxA_2 and, because of the inability of the anuclear platelet to generate more COX, will do so for the life of the platelet despite aspirin's short plasma half-life of only 20 min (15).

Some controversy surrounds the optimal dose of aspirin. It seems, however, that doses in the range of 30–1500 mg/day are comparably effective in providing cardiovascular benefit, with higher doses producing more adverse gastrointestinal side-effects (16). Aspirin, when used for therapy against arterial thrombosis, is commonly given at doses of 81–325 mg orally once daily and is generally well tolerated. The clinical importance of the anti-inflammatory properties of aspirin and the optimal dose needed to minimize the markers of inflammation remain controversial.

Thienopyridines

The thienopyridines ticlopidine and clopidogrel irreversibly inhibit platelet aggregation by preventing ADP-mediated structural alterations in the GP IIb/IIIa receptor, thereby inhibiting platelet binding to fibrinogen (17). Aspirin synergistically potentiates the antiaggregating and antithrombotic effects of these agents (18).

Despite their structural similarity, ticlopidine and clopidogrel have a number of distinct and important differences. The clinically relevant effects of ticlopidine require treatment for 2–3 days, and the drug requires up to 7 days after a loading dose to demonstrate its full antiplatelet effect (19). With the appropriate loading dose, clopidogrel attains near-maximal levels of platelet inhibition in control subjects in only 5 h (20). This pharmacodynamic edge of clopidogrel may theoretically translate into improved outcomes compared with ticlopidine when these agents are dosed for acute coronary syndromes and urgent procedures.

Life-threatening thrombotic thrombocytopenia purpura (TTP) has been reported with the use of both of these agents, although the incidence appears to be considerably higher with ticlopidine. In stented patients treated with ticlopidine, TTP was reported to occur in 1 in every 1600 (21) to 4800 patients (22). In a recent publication, 11 cases of TTP associated with clopidogrel were identified among over 3 million patients treated (23).

Other blood dyscrasias, such as neutropenia, and minor adverse effects tend to be far more common with ticlopidine and lead to the discontinuation of the drug in approximately 20% of patients (24). On the other hand, clopidogrel is equally well-tolerated as aspirin (25).

Platelet glycoprotein IIb/IIIa inhibitors

TxA_2 and ADP, inhibited by aspirin and the thienopyridines, respectively, represent only a fraction of the agonists known to stimulate platelet activation and aggregation. Once researchers elucidated the GP IIb/IIIa platelet surface protein as the platelet receptor ultimately responsible for platelet-to-platelet adherence, the final common pathway for platelet aggregation irrespective of the initiating agonists, blockade of this receptor became an enticing prospect.

The first GP IIb/IIIa inhibitor to be clinically evaluated was abciximab, a Fab fragment of a chimeric human/murine monoclonal antibody to the GP IIb/IIIa receptor. Abciximab is a potent inhibitor of platelet aggregation that demonstrates receptor binding up to 2 weeks after a 12-h infusion (26). Platelet recovery occurs only with the production of new platelets from the marrow. Abciximab is not specific to the GP IIb/IIIa receptor and also shows affinity for the platelet vitronectin receptor, a structure that plays a role in cell adhesion, migration and proliferation, although the clinical significance of this is not yet clear.

Two other agents in this category have been approved for use in the USA. Eptifibatide is a synthetic peptide the development of which was inspired by a protein found in the venom of the Southeastern Pygmy rattlesnake. The onset of antiplatelet effect occurs within minutes of intravenous dosing of eptifibatide and abates within 2–3 h following cessation of therapy. Tirofiban is a nonpeptide tyrosine analogue with a likewise venomous heritage and similarly rapid pharmacodynamics. Unlike abciximab, both of these drugs are highly specific competitive inhibitors of the GP IIb/IIIa receptor and show no affinity for the vitronectin receptor.

All the GP IIb/IIIa inhibitors are available in the intravenous form only and are thus limited to use in the hospital setting. Oral drugs in this class have been widely tested but none have shown clinical benefit. Indeed, in many trials treatment with oral GP IIb/IIIa inhibitors has resulted in increased adverse outcomes when compared with standard therapy (27, 28).

Other antiplatelet agents

Many other drugs possess antiplatelet activity including dipyridamole, NSAIDs (nonsteroidal anti-inflammatory drugs), dextran, sulfinpyrazone and cilostazol. Currently, these agents find little application in the treatment of cardiac disease and will not be discussed. Notably, however, a combination of dipyridamole and aspirin has recently been approved for the secondary prevention of cerebrovascular events (29).

Clinical applications

Primary prevention

Aspirin. Aspirin has been well tested in the prevention of cardiovascular events in patients with no history of myocardial infarction. Two large prospective randomized trials have established that aspirin provides some benefit when used empirically in large populations of generally healthy patients while at the same time casting doubts on the wisdom of such a nonselective approach. Both the British Doctors' Trial (30) and the Physicians' Health Study (31) enrolled and randomized male physicians to treatment with aspirin or no aspirin. The smaller British trial (5139 subjects), in which patients took 500 mg of aspirin daily, showed no significant difference in mortality between the two cohorts and demonstrated an increased incidence of disabling stroke among those on aspirin (19.1% vs 7.4%, $P < 0.05$). The Physicians' Health study enrolled four times as many persons and produced more encouraging results. There was a 44% ($P < 0.00001$) reduction in the risk of myocardial infarction in the group taking 325 mg of aspirin every other day compared with the placebo group. There was no reduction in cardiovascular mortality in the aspirin group, but also no significant increase in stroke. Further analysis of the data revealed that only

persons older than 50 years derived benefit from aspirin therapy.

Aspirin therapy for primary prevention in persons with known cardiovascular risk factors was addressed in the Hypertension Optimal Treatment trial (32). In this randomized study, patients with hypertension who ingested 75 mg of aspirin daily enjoyed a 15% reduction in major cardiovascular events ($P = 0.03$) and a 36% decrease in the rate of myocardial infarction ($P = 0.002$). There was a nonsignificant trend toward lower mortality and no increase in stroke in the aspirin cohort. The Thrombosis Prevention Trial, in which men with cardiovascular risk factors were randomized to 75 mg aspirin daily, reinforced these positive results when it showed a 20% reduction in major cardiac events at 6 years ($P = 0.04$) (33).

Based on these data, low-dose aspirin appears to be effective in the primary prevention of myocardial infarction but does not seem to have a detectable impact on mortality in this population. Persons who are older, who have risk factors for coronary disease, or who have stable angina are more likely to benefit from aspirin therapy (Table 1). The low death rate in the relatively healthy populations studied may explain the perceived lack of mortality benefit in these trials.

Ticlopidine/clopidogrel. There are no data addressing the role of these agents in primary prevention.

Secondary prevention

Aspirin. Long-term aspirin therapy in patients who have already suffered a herald thrombotic event, myocardial infarction, stroke, or peripheral embolism, results in a significant reduction in disease recurrence. To date, the most extensive compilation of data in this category is the Antiplatelet Trialists' meta-analysis that reviewed the results of 145 individual aspirin trials comprising about 100 000 patients (16). About 70% of these patients were determined to be 'high-risk', with a history of myocardial infarction, angina, prior revascularization and stroke. In this group, chronic therapy with aspirin cut the rate of nonfatal myocardial infarction by 35% and vascular death by 18%. More specifically, in the roughly 20 000 patients who had suffered a prior myocardial infarction, the reduction in the composite end-point of myocardial infarction, stroke, or vascular death was a significant 25%. Based on data such as these, aspirin has become an assented addition to the pharmaceutical regimen of patients with established vascular disease.

Table 1. Gradient of benefit in trials of aspirin therapy.

Indication for therapy	Magnitude of benefit
Primary prevention	
No CAD risk factors	4 events/1000 men treated for 5 years
CAD risk factors	5 events/1000 men treated for 1 year
Patients with angina	51 events/1000 patients treated for 4 years
Secondary prevention (after AMI)	36 events/1000 patients treated for 2 years
Treatment of unstable angina	50 events/1000 patients treated for 6 months
Treatment of AMI	24 deaths/1000 patients treated for 5 weeks

AMI, acute myocardial infarction; CAD, coronary artery disease. (Adapted from (13) with permission.)

Ticlopidine/Clopidogrel. Despite the limited research in the use of the thienopyridines for secondary prevention after vascular events, several well-designed studies have demonstrated that these drugs are extremely effective in this clinical province. In the Canadian American Ticlopidine Study, which followed 1072 patients with prior transient ischaemic attack or stroke for a mean of 24 months, treatment with ticlopidine reduced the risk of stroke, myocardial infarction, or vascular death by 30.5% (95% CI 7.5–48.3%; $P = 0.006$) when compared with placebo (24). Another study compared ticlopidine with aspirin in the same demographic cohort and showed that ticlopidine had a narrowly significant advantage in the prevention of death or nonfatal stroke (RRR 12%, 95% CI –2 to 26%; $P = 0.048$) (34).

A considerably larger project studied 19 185 patients with atherosclerotic vascular disease manifested by prior stroke, myocardial infarction, or symptomatic peripheral vascular disease (25). Participants were randomized to clopidogrel 75 mg or aspirin 325 mg daily and were followed for 1–3 years for recurrent vascular events. Patients treated with clopidogrel fared slightly better with a relative reduction of 8.7% (95% CI 0.3–16.5, $P = 0.043$) in the annual incidence of ischaemic stroke, myocardial infarction, or vascular death.

Taken together, these studies reveal that the thienopyridines are at least as good as aspirin in the secondary prevention of vascular events. Because of the higher cost of these drugs and the additional adverse effects of ticlopidine, thienopyridines should be employed as second-line therapy.

Unstable angina

Unstable angina and non-Q-wave myocardical infarction are closely positioned along the spectrum of acute ischaemic syndromes. As they share the common physiology of atheromatous plaque rupture or fissure with thrombus formation, but typically without vessel occlusion as is seen with ST-elevation myocardial infarction, and because they are often indistinguishable

in the acute setting, these two entities are often considered together. The fact that patients suffering an acute coronary syndrome that manifests as ST-segment depression have a greater 6-month mortality than those presenting with an ST-segment-elevation myocardial infarction justifies aggressive therapy in these patients (35).

Aspirin. Four trials comprising 2579 patients with unstable angina showed uniform benefit from aspirin therapy compared with placebo (36–39). Patients treated with 75–1200 mg of aspirin daily had a mortality that was half of that seen in the placebo cohort. In the largest of these studies the mortality improvement persisted up to a year after only 3 months of drug therapy (36).

Ticlopidine/Clopidogrel. In an open trial of 542 patients with unstable angina, ticlopidine reduced the incidence of vascular death and nonfatal myocardial infarction by 46% ($P = 0.009$) when compared with a therapy that did not include heparin or aspirin (40). The fact that no clinical difference was evident in the groups until after 15 days may be explained by the delayed onset of antiplatelet activity with ticlopidine. Clopidogrel has not been studied in this population, and there have been no trials comparing these agents to aspirin. The ongoing CURE study (Clopidogrel in Unstable angina to prevent Recurrent ischaemic Events), which will compare aspirin with the combination of aspirin plus clopidogrel in over 10 000 patients, should provide useful information regarding the use of these agents in acute coronary syndrome. At this time, these drugs are used for unstable angina only in patients who cannot tolerate aspirin.

Platelet glycoprotein IIb/IIIa receptor inhibitors. The role of these intravenous medications in acute coronary syndromes is being investigated energetically. Four large trials with a total of over 18 000 patients have produced some encouraging results (41–44). The pooled results of these studies show that the drugs tirofiban, eptifibatide and lamifiban (not available) prevented the combined end-point of death or myocardial infarction in 15–32 of every 1000 patients treated (45). Aspirin and heparin served as adjunctive therapy in each of these trials, and percutaneous revascularization was discouraged.

Several pertinent findings deserve further comment. The clinical efficacy of GP IIb/IIIa inhibition was incremental to that achieved with aspirin and heparin; but despite enhanced platelet inhibition, no increased risk of intracranial haemorrhage was observed in these trials. Patients who proceeded to have percutaneous revascularization were found to benefit most from therapy. Finally, the addition of heparin proved to be a key component to therapy. In the PRISM-PLUS study, tirofiban alone (without heparin) was linked to increased mortality at 1 week, a finding that led to the premature discontinuation of the no-heparin arm of the protocol (41).

Abciximab has been studied in the setting of acute coronary syndromes, but only as an adjunct to percutaneous intervention (46). The recently completed GUSTO IV-ACS trial studied the role of a prolonged (24- or 48-h) infusion of abciximab in persons with unstable angina and non-Q-wave myocardial infarction, but results are not yet available. Tirofiban and eptifibatide carry an indication from the Food and Drug Administration for use in acute coronary syndromes regardless of whether the patient is to be managed medically or with coronary revascularization.

Acute myocardial infarction

Aspirin. The 1996 *American College of Cardiology/American Heart Association Guidelines for the Management of Patients with Acute Myocardial Infarction* recommends aspirin acutely in all patients suffering a myocardial infarction (47). 'A dose of 160 to 325 mg should be given on day one of acute myocardial infarction and continued indefinitely on a daily basis thereafter.' This approbation is made on the basis of overwhelming evidence of the benefit of aspirin in heart attack victims.

The most compelling data for the benefit of aspirin therapy for the treatment of heart attack came out of the Second International Study of Infarct Survival (12). This trial randomized 17 187 patients with suspected myocardial infarction to one of four treatment groups: streptokinase alone, aspirin alone (160 mg daily for 30 days), both drugs, or neither. During the 5 weeks of clinical evaluation, treatment with aspirin reduced mortality irrespective of whether patients were allocated to the streptokinase or placebo infusions (25% and 21%, respectively; each $P < 0.001$). This translates to 25 deaths averted for every 1000 patients treated with aspirin. Remarkably, therapy with aspirin alone resulted in the same mortality benefit as therapy with streptokinase alone (Fig 2). The patients treated with both drugs enjoyed the most favourable outcome with a mortality that was 42% lower than in the control group ($P < 0.00001$). A 10-year follow-up evaluation of this population revealed that the mortality improvement with aspirin persisted over time (48).

The additive effect of aspirin and thrombolytics has been documented in over two dozen trials in addition to ISIS-2 (49). The inclusion of aspirin to a thrombolytic regimen does not increase the acute bleeding risk (12). A practical approach for the use of this drug would be to administer 160–325 mg of

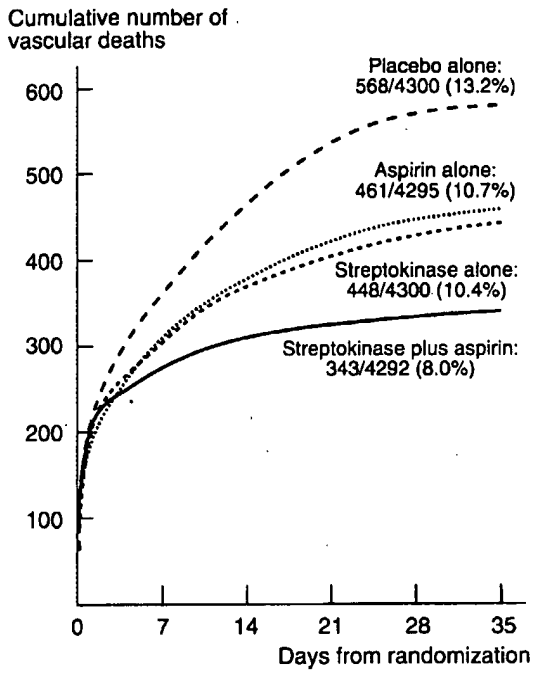

Figure 2. Cumulative vascular mortality in days 0–35 for patients with acute myocardial infarction treated with placebo, aspirin alone, streptokinase alone, or aspirin plus streptokinase. Data are from the Second International Study of Infarct Survival (ISIS-2). The difference between the aspirin and streptokinase groups did not achieve conventional statistical significance. (Reproduced from (12) with permission.)

chewable aspirin as early as possible into the course of an acute myocardial infarction as this form is more quickly absorbed (47).

Ticlopidine/Clopidogrel. No large-scale studies addressing the utility of the thienopyridines in the acute treatment of ST-segment-elevation myocardial infarction have been published, and therefore these agents are currently not recommended for this use. Limited data suggest that a benefit could be gained with these medications. In a subset of the CAPRIE trial, there was a trend toward mortality improvement (RRR 7.4%, 95% CI –5.2 to 18.6) with clopidogrel in patients who had suffered a myocardial infarction within the preceding 35 days (25). The ongoing COMMIT trial, being carried out in China, will randomize 30 000 patients with an acute ST-segment-elevation myocardial infarction to either aspirin or the combination of aspirin and clopidogrel.

Platelet glycoprotein IIb/IIIa inhibitors. The current strategy for the treatment of ST-segment-elevation myocardial infarctions is early reperfusion of the obstructed coronary vessel. Patients who fail to reperfuse infarcting myocardium have a three-fold increase in mortality (50). While thrombolytic therapy has had a substantial impact on survival, the bleak fact remains that only half of all patients receiving thrombolytic agents actually achieve complete reperfusion of the infarct-related artery. This is at least partially the result of the inability of fibrinolytic therapy to dissolve platelet-rich thrombi, the type of clot that is most frequently found in persons who are resistant to thrombolytics (51). Furthermore, platelets may secrete substances that inhibit plasminogen activator and at least partially neutralize the effects of thrombolytics (52).

Pilot studies evaluating strategies for combining thrombolytic and platelet inhibiting therapy in the acute stages of myocardial infarction have shown good success. The angiographic results of the Thrombolysis In Myocardial Infarction (TIMI) 14 trial showed significantly improved infarct vessel patency at 60 and 90 min with the combination of half-dose alteplase (recombinant tissue plasminogen activator) and abciximab (53). When very low doses of heparin were used in this group, the patients treated with the alteplase–abciximab combination actually had a lower bleeding incidence than patients treated with conventional doses of thrombolytic agent. Similar results from the Strategies for Patency Enhancement in the Emergency Department (SPEED) group bolstered the case for combination therapy (54). The results of these pilot studies led to the currently enrolling GUSTO-IV AMI trial. This is a 14 000-patient international protocol that will evaluate full-dose reteplase compared with half-dose reteplase plus abciximab bolus and infusion in myocardial infarction.

Percutaneous coronary intervention

Percutaneous transluminal coronary angioplasty (PTCA), with or without adjunctive coronary artery stenting, has become an effective alternative to coronary artery bypass graft surgery in the treatment of coronary stenosis. Late (3–6 month) restenosis of the target vessel often limits the clinical success of PTCA. The addition of a stent to an artery that has been suboptimally dilated with PTCA decreases the rate of restenosis by increasing the size of the initial luminal diameter. Placing a foreign metal object into a diseased vessel, however, increases the risk of thrombus formation at the site. The clinical entity of acute (< 24-h) or subacute (1- to 14-day) thrombosis of the stented segment frequently accompanied stent placement prior to the routine use of antiplatelet medication. The incidence of this catastrophic event was as high as 24% with a mortality of 32% in early studies (55). In recent years, the increasing use of antiplatelet drugs has greatly enhanced the success rate of percutaneous coronary interventions.

Aspirin. Aspirin has essentially come to be a mandatory therapy for patients undergoing percutaneous coronary intervention. Schwartz and co-workers demonstrated a 78% relative reduction in the occurrence of periprocedural Q-wave myocardial infarction with a combination of aspirin and dipyridamole (56). The routine use of dipyridamole fell out of favour when it was shown to offer no incremental benefit to aspirin (57). In a blinded review of 300 consecutive patients who underwent PTCA, Barnathan and co-workers found that patients who received no aspirin were almost six times more likely to suffer clinically relevant target-vessel thrombosis ($P = 0.05$) (58).

Ticlopidine/Clopidogrel. The addition of a metal stent to diseased and disrupted coronary endothelium dramatically increases the risk of early thrombus formation with its attendant morbidity. The blockade of ADP-dependent platelet activation potentiates the antiplatelet activity of aspirin and reduces *in vitro* thrombus formation in plasma of patients undergoing coronary stenting (59).

Compelling evidence in support of thienopyridine therapy comes from four randomized trials that compared ticlopidine to conventional anticoagulation in the setting of coronary stenting (Table 2). In these studies, the combination of ticlopidine and aspirin resulted in significantly lower rates of early thrombosis and cardiac events when compared with anticoagulation alone (61, 62) or anticoagulation plus aspirin (63, 64).

Consistent with the delayed onset of antiplatelet activity with ticlopidine, each of these studies demonstrates a 24–72-h lag in the onset of benefit derived

Table 2. Prevention of major cardiac events after coronary artery stent insertion: ticlopidine compared with anticoagulant therapy. Cardiac events included death due to cardiac causes, myocardial infarction, repeat percutaneous transluminal coronary angioplasty of the stented vessel, or bypass surgery at 30 days. In the FANTASTIC study, cardiac events were secondary end-points.

Study	*n*	Population risk	Absolute reduction in cardiac events, %	*P*
FANTASTIC	485	Mixed	2.6	0.37
MATTIS	350	High	5.4	0.07
ISAR	517	Mixed	4.6	0.01
STARS	1652	Low	1.9	< 0.05

FANTASTIC, Full Anticoagulation versus ASpirin and TIClopidine after stent implantation; ISAR, Intracoronary Stenting and Antithrombotic Regimen; MATTIS, Multicenter Aspirin and Ticlopidine Trial after Intracoronary Stenting; STARS, STent Anticoagulation Restenosis Study. (Reproduced from (60) with permission.)

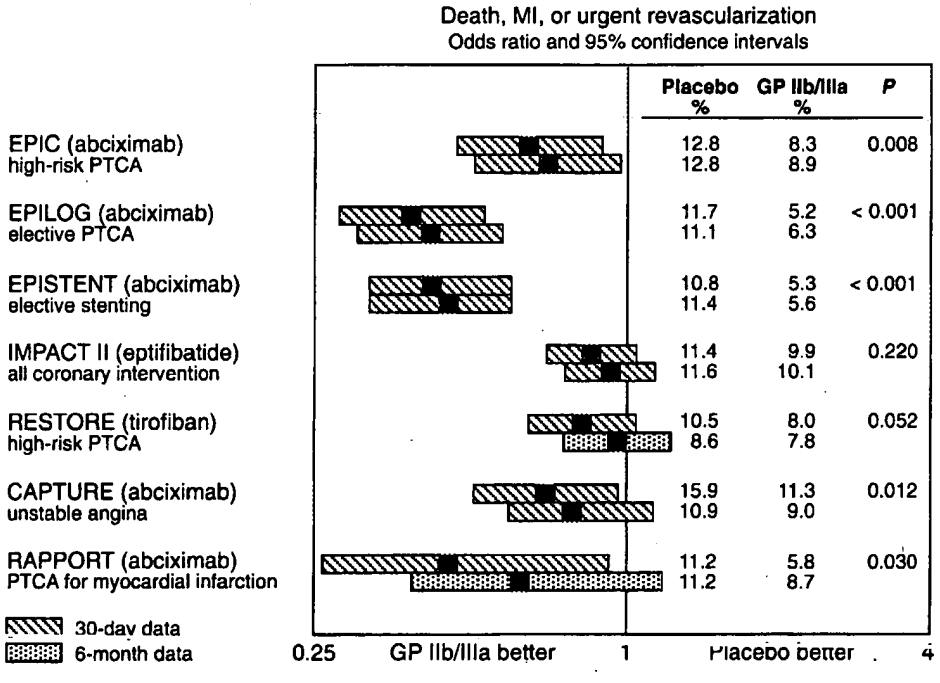

Figure 3. Composite 30-day end-point rates for death, myocardial infarction (MI) or urgent repeat revascularization and 6-month end-point rates for death or MI for the GP IIb/IIIa interventional trials. CAPTURE, C7E3 AntiPlatelet Therapy in Unstable REfractory angina; EPIC, Evaluation of c7E3 for Prevention of Ischemic Complications; EPILOG, Evaluation in PTCA to Improve Long-term Outcome with abciximab GP IIb/IIIa blockade; EPISTENT, Evaluation of Platelet Inhibition in STENTing; IMPACT, Integrilin to Minimize Platelet Aggregation and Coronary Thrombosis. (Adapted from (45) with permission.)

Drugs that inhibit platelet function improve short- and long-term outcomes significantly when used for primary or secondary prevention of cardiac events, for the treatment of acute coronary syncromes or as adjunctive therapy with percutaneous coronary interventions.

from thienopyridine treatment when compared with standard therapy. Ticlopidine was dosed in the cardiac catheterization laboratory (63), immediately after the procedure (62, 64), or upon randomization within 6 h of stenting (61), but was never administered early enough to ensure adequate platelet inhibition at the time of stent deployment. Pretreatment with ticlopidine, or the use of clopidogrel, with the rapid effect that a loading dose of 300 mg provides (65), may have produced better early outcomes.

To date there has been no large, multicentre, randomized series published of stenting in patients treated with clopidogrel. Two small, randomized studies comparing clopidogrel with ticlopidine found no significant difference in the incidence of major adverse cardiac events in the two groups (66, 67). In the recently completed CLASSICS (CLopidogrel ASpirin Stent International Cooperative Study), 1020 patients undergoing stent implantation were randomized for 28 days to ticlopidine or clopidogrel (68). In this study, clopidogrel had a more favourable side-effect profile than ticlopidine, and the cardiac event rates were low and comparable in both groups.

Platelet glycoprotein IIb/IIIa inhibitors. Several large-scale trials have assessed the use of GP IIb/IIIa inhibitors in patients undergoing percutaneous coronary interventions. The various settings of these studies include elective PTCA (69–71), primary PTCA for acute myocardial infarction (71–74), high-risk PTCA (73, 74), PTCA for unstable angina (46, 71, 73, 74) and stenting (70). All trials were randomized and blinded. Aspirin therapy was included in each protocol, and the patients who underwent stenting received ticlopidine. The dosing and duration of the intravenous GP IIb/IIIa inhibitor varied based on the pharmacological characteristics of the drugs. Tirofiban and eptifibatide, with a relatively brief duration of action, were infused for a longer period of time (24–36 h) than abciximab (12 h).

The clinical experience with GP IIb/IIIa inhibitors in the setting of percutaneous coronary intervention is summarized in Figure 3. Based on the favourable results seen in EPIC, EPILOG and EPISTENT, therapy with abciximab appears to provide enhanced protection from ischaemic complications when compared with the other agents. The trend toward lower events in the groups treated with eptifibatide and tirofiban failed to attain conventional statistical significance at either the 30-day or 6-month mark.

Although significant bleeding was observed particularly in the EPIC trial, the subsequent use of low-dose, weight-adjusted procedural heparin (70 U/kg), avoidance of postprocedural heparin, and early vascular sheath removal has resulted in a bleeding risk equivalent to that seen in patients treated with heparin alone. None of the studies of this drug class suggested that GP IIb/IIIa inhibitor therapy increases haemorrhagic cerebrovascular events.

Platelet reactivity before procedure has been reported to be an important risk factor in the development of restenosis after PTCA (75). While it is evident that GP IIb/IIIa inhibition at the time of coronary intervention decreases early thrombotic complications, it is unclear whether platelet blockade with these drugs impedes target-vessel restenosis. A lower rate of revascularization was seen in EPIC as well as in the diabetic subgroup of EPISTENT, but these results were not reproduced in EPILOG. The only randomized study to employ intravascular ultrasound to assess vessel patency at 6 months revealed no significant difference between groups allocated to abciximab and placebo based on measurement of the median luminal diameter of the stented segment (76).

Conclusion

Pharmacological platelet inhibition has emerged as a cornerstone of therapy against inappropriate thrombus formation. Drugs that inhibit platelet function significantly improve short- and long-term outcomes when used for primary and secondary prevention of cardiac events, in the treatment of acute coronary syndromes, and as adjunctive therapy with percutaneous coronary interventions. Clinical research into therapy with antiplatelet medication is currently an extremely active field and will likely lead to a substantial change in the current treatment of cardiovascular disease.

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Plasma Levels of Tumor Necrosis Factor and Endothelial Response in Patients with Chronic Arterial Obstructive Disease or Raynaud's Phenomenon

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ABSTRACT

Tumor necrosis factor alpha (TNF-alpha) is a cytokine that affects endothelial cells' function by changing their antithrombotic potential to a net procoagulant effect. Only a few data have so far been reported for the pathophysiologic role of TNF in vascular diseases in the involvement of microvessels and/or macrovessels and a prothrombotic state. In the present study the authors evaluated plasma TNF (and interleukin-1) levels in 20 patients with chronic arterial obstructive disease (CAOD) with intermittent claudication and 10 CAOD patients with more severe disease (pain at rest/skin ulcers). In addition, they studied 10 patients with Raynaud's phenomenon (RP), suspected to be secondary to a collagen disease. The control group consisted of 20 subjects matched for sex and age with the three groups of patients. TNF levels were assayed by enzyme-linked immunosorbent assay.

The antigen levels of von Willebrand factor (vWF), tissue plasminogen activator (t-PA), and its inhibitor (PAI) were also determined as markers of release from the endothelium, while the fragment 1+2 of prothrombin (F1+2) and thrombin-antithrombin III (TAT) complexes were assessed as indexes of systemic thrombin generation.

TNF levels were significantly higher in both groups of CAOD patients than in controls or RP patients, and the same was true for vWF. t-PA was significantly higher only in the CAOD subjects with more severe disease. No differences among groups were seen in PAI antigen/activity or thrombin generation. When data were corrected for age, TNF no longer differentiated CAOD patients from controls and RP subjects. There were strong direct correlations between TNF and age (r=0.57, P=0.0001); TNF and t-PA (r=0.43, P=0.002); and TNF and vWF (r=0.52, P=0.0001). The association of TNF with vWF was independent of other variables. The present study suggests that TNF plays an important role in the pathophysiology of arterial diseases of atherosclerotic origin, especially during aging.

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Introduction

Abnormal endothelial responses in patients with Raynaud's phenomenon (RP) have been reported by several groups, suggesting that there is an imbalance between antithrombotic and prothrombotic mediators released from endothelial cells. Kahaleh[1] first described the abnormally high levels of von Willebrand factor (vWF) in these patients. More recently our group[2] showed that subjects with RP suspected to be secondary to a collagen disease or with scleroderma-associated RP have reduced fibrinolytic potential, owing to a lack of increase in tissue plasminogen activator (t-PA) after venous occlusion, in parallel with persistence of high plasminogen activator inhibitor (PAI) levels. We also found lower plasma protein C activity in the same subjects.[3]

To learn more about the mechanisms leading to these prothrombotic modifications, we have investigated the possible roles of two monocyte-derived peptides, the endothelium-targeted cytokine tumor necrosis factor alpha (TNF alpha) and interleukin-1 beta (IL-1 beta).[4-6] We measured the plasma levels of TNF alpha and IL-1 beta in patients with isolated RP suspected to be secondary to a collagen disease, which is a clinical example of microvascular involvement. For comparison, we measured the same parameters in subjects also showing damage to large vessels of atherosclerotic origin, still characterized by a prothrombotic tendency[7,8] such as patients with chronic arterial obstructive disease (CAOD) who have intermittent claudication or leg pain at rest/skin ulcers. In addition to TNF and IL-1, we also measured plasma levels of the prothrombotic substances, such as t-PA, PAI, vWF, reported to be secreted or released from endothelium by the action of the cytokines.[9,10] Systemic thrombin generation was monitored by measuring plasma levels of the cleavage fragment 1+2 of prothrombin (F1+2) and the circulating thrombin-antithrombin III (TAT) complexes.

Patients and Methods

Ten patients (3 men, 7 women; mean age 40.1 ±8.3 years) with isolated RP were studied during the winter of 1991–1992. Diagnosis was based on clinical history and photoplethysmography patterns in response to cold stimuli.[11] The diagnosis of underlying connective tissue disorder was ruled out according to the American Rheumatism Association criteria. The etiologic investigation included assay for antinuclear antibodies and biomicroscopy of the fingernail fold, as described elsewhere.[12]

Twenty nondiabetic patients (17 men, 3 women; mean age 67.6 ±6.8 years) with intermittent claudication (Fontaine stage II) were also studied. Diagnosis was based on the clinical history of leg pain on walking (resolving in a few minutes on standing) for at least six months, plus an ankle/arm systolic blood pressure index (API) < 0.80. The absolute walking distance in the treadmill test ranged between 52 and 470 meters (mean 155.6 ±115.9). Fifteen patients were cigarette smokers.

Finally, 10 patients (7 men, 3 women; mean age 72.7 ±7.5 years) with more severe limb ischemia (Fontaine stage III/IV), with pain at rest or skin ulcers (8 of them smokers), entered the study. As for the previous group, diabetic subjects and cancer patients were excluded. In addition, all patients enrolled were free of infections at the time of the study.

Because of differences in age between RP patients and those with CAOD, the control group consisted of 20 healthy subjects (13 men, 7 women) with a wide range of age, max sixty-eight, min twenty-one years (mean 41.9 ±12). Informed consent was obtained from all the participants.

Blood Tests

Blood was drawn from an antecubital vein between 8.00 and 9.00 AM because of the circadian variation of fibrinolytic components. Citrated plasma was prepared immediately after collection by centrifugation at 300 × g for twenty minutes at 4°C. Plasma aliquots were stored frozen at –80°C until assayed.

Plasma Il-1 assay was performed by commercially available immunometric method. Data were expressed in pg/mL. Plasma TNF assays were performed with a commercially available technique. The results were expressed in pg/mL. Plasma t-PA antigen and PAI activity were determined with commercially available kits, and the results were expressed as ng/mL (tPA antigen) or as arbitrary units (AU/mL, PAI activity); PAI-1 antigen in plasma, expressed as ng/mL, was assayed with a commercially available enzyme-linked immunosorbent assay (ELISA) kit. vWF antigen levels were also measured with a commercial ELISA kit and

expressed as U/mL. The prothrombin fragment F1+2 and the thrombin-antithrombin III (TAT) complexes in plasma were determined by commercially available immunoassays and expressed as nmol/L or μg/L.

Data Presentation and Statistical Analysis

Data are presented as means ±standard deviations (SD). Analyses of variance (and of covariance) with the Duncan multiple comparisons test (or Bonferroni adjustment) were applied when appropriate. Pearson's product moment coefficients were calculated to assess relations between variables. Multiple regression according to maximum R-square improvement method was also employed to assess the independence of associations.

Results

Table I reports all the parameters studied for each group. As can be seen, TNF values from CAOD patients with both intermittent claudication and leg pain at rest were significantly higher than those for RP patients and controls. The same was true for vWF levels, while t-PA levels were significantly higher only in CAOD subjects with more severe disease than in controls or RP or CAOD patients with intermittent claudication.

We also investigated the relationships between TNF and IL-1 and the coagulation/fibrinolysis indexes, including age, in the analysis, because of wide scatter of ages of the subjects (Table II). There were significant correlations between age and TNF, vWF, and t-PA. In addition, TNF levels were correlated with vWF and t-PA, with these latter two parameters strictly related to each other. TNF was also correlated with IL-1. The two markers of thrombin generation, F1+2 and TAT, were significantly correlated. We also confirmed the direct association between t-PA antigen and PAI activity. When data corrected for age were analyzed, TNF no longer differentiated patients with CAOD from RP subjects and controls, while vWF still did (Fig. 1), and t-PA remained significantly higher in CAOD patients with pain at rest only when compared with CAOD patients with intermittent claudication (data not shown).

The independence of the associations shown in Table II was assessed by multiple regression analysis. With TNF taken as the dependent variable, only the correlations between age and vWF remained significant (Table III).

Discussion

The effects of IL-1 and TNF on endothelial cells involve both proinflammatory and procoagulant responses.[13,14] van der Poll et al[15] demonstrated that a single injection of 50 μg/m^2 TNF elicits a procoagulant response in normal subjects through activation of the "common pathway." Bauer et al[16] have shown that there is a significant increase in the indirect indexes of thrombin generation in plasma when cancer patients are infused intravenously with recombinant TNF and that there is a threshold dose of TNF at which this cytokine acts on coagulation processes. The fibrinolytic system is also affected by TNF. van Hinsbergh et al[17] confirmed previous in vitro observations[18] demonstrating increases in both PAI-1 and t-PA antigen after infusion of TNF into cancer patients with active disease. Finally, the plasma levels of vWF, a large multimeric glycoprotein released and secreted by endothelial cells under several stimuli (including thrombin), are significantly increased in healthy humans after TNF infusion.[19]

The above-mentioned data, obtained from a variety of patients and healthy controls, are partly in agreement with our present data, the first for this clinical model, and elevated levels of circulating TNF (and/or IL-1) have been described in patients with infections,[20,21] severe chronic heart failure,[22] and several connective tissue diseases[23,24] and scleroderma[25] often associated with RP.

We emphasize the importance of age that emerges from our data, which has never before been mentioned. The direct relationship between TNF and age is, in fact, responsible for the disappearance of differences between patients and controls after analysis of covariance (correction for age) was applied to the TNF data. We emphasize here the direct relationship between TNF and vWF still seen after multiple regression analysis (when t-PA had ceased to be associated with TNF). vWF (and to some extent t-PA) differentiates patients with both less and more severe CAOD from controls. The same was not true for RP patients, even though in previous reports, as mentioned, VWF and t-PA were described as elevated in these patients, too.

Moreover, neither vWF nor t-PA synthesis seems to be directly related to TNF action, according to in vitro and ex vivo experiments, although the release can be modulated by cytokines in response to other stimuli. One of these might

Table I

TNF, IL-1, and Coagulation/Fibrinolysis Parameters Values in Plasma from Controls and Patients. Means ±SD with Asterisk on Same Side (Upper/Lower) Are Not Statistically Different According to ANOVA (Duncan Comparisons)

	Controls (n=20)	RP (n=10)	CAOD II (n=20)	CAOD III/IV (n=10)
TNF α (pg/mL) F=6.76 P=0.0006	7.19 ±10.88 *	7.73 ±7.55 *	17.39 ±12.14 *	23.72 ±11.70 *
IL-1β (pg/mL) F=0.92 P=04	11.09 ±7.55	8.45 ±11.49	13.76 ±13.03	5.9 ±8.11
PAI-1 Ag (ng/mL) F=0.47 P=0.7	39.29 ±14.44	35.38 ±19.91	44.8 ±22.63	39.5 ±27.57
t-PA Ag (ng/mL) F=6.7 P=0.0008	5.42 ±4.24 *	6.07 ±3.83 *	9.18 ±4.73 *	14.74 ±7.38 *
PAI Act (Au/mL) F=2.41 P=0.07	9.26 ±6.43	6.68 ±4.27	3.35 ±3.80	7.63 ±10.26
vWF (U/mL) F=16.21 P=0.0001	0.75 ±0.27 *	0.96 ±0.49 *	1.59 ±0.50 *	1.97 ±0.85 *
F1+2 (nmol/L) F=16.5 P=0.19	1.10 ±0.16	2.92 ±5.28	0.98 ±0.36	1.60 ±0.64
TAT (mg/L) F=4.47 P=0.005	2.12 ±2.24 *	8.88 ±9.33 *	3.03 ±1.98 *	5.78 ±6.89 *

Legend: RP=Raynaud's phenomenon, CAOD II=chronic arterial obstructive disease with intermittent claudication (Fontaine stage II), CAOD III/IV=chronic arterial obstructive disease with pain at rest/skin ulcers (Fontaine stage III/IV), TNF α=tumor necrosis factor-alpha, IL-1β=interleukin-1 beta, PAI-1 Ag=plasminogen activator inhibitor-1 antigen, t-PA Ag=tissue plasminogen activator antigen, PAI Act=plasminogen activator inhibitor activity, vWF=von Willebrand factor, F1+2=fragment 1+2 of prothrombin, TAT=thrombin-antithrombin III.

Table II

Correlations Between TNF, IL-1 and Coagulation/Fibrinolysis Parameters in Controls and Patients (n=58)

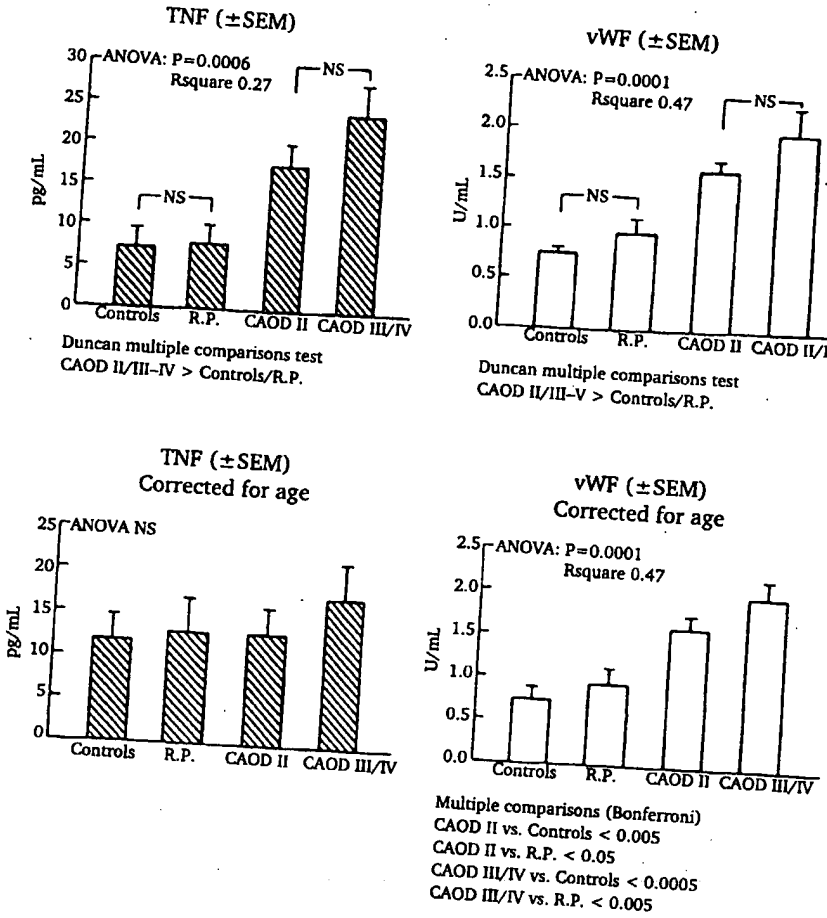
	Age	TNF	t-PA	vWF	IL-1	PAI Ag	PAI Act	F1+2	TAT
Age	–	r=0.57 P=0.0001	r=0.48 P=0004	r=0.55 P=0001	r=0.10 NS	r=0.003 NS	r=0.19 NS	r=0.20 NS	r=0.15 NS
TNF	–	–	r=0.43 P=0.002	r=0.52 P=0.0001	r=0.49 P=0.0001	r=0.24 P=0.09	r=0.07 NS	r=–0.03 NS	r=–0.00 NS
t-PA	–	–	–	r=0.67 P=0.0001	r=0.14 NS	r=0.20 NS	r=0.35 P=0.01	r=0.00 NS	r=–0.08 NS
vWF	–	–	–	–	r=0.15 NS	r=0.23 NS	r=0.17 NS	r=0.20 NS	r=0.13 NS
IL-1	–	–	–	–	–	r=0.07 NS	r=–0.03 NS	r=0.14 NS	r=–0.18 NS
PAI Ag	–	–	–	–	–	–	r=0.41 P=0.002	r=0.35 P=0.01	r=0.17 NS
PAI Act	–	–	–	–	–	–	–	r=0.12 NS	r=0.39 P=0.005
F1+2	–	–	–	–	–	–	–	–	r=0.55 P=0.001
TAT									

Legend: See Table I.

Table III

Parameters Estimated by Multiple Regression (Maximum R-Square Improvement). Dependent Variable: TNF Best 3-Variable Model

	Coefficient (β)	SEM	F	P
Age	0.28	0.08	11.19	0.0017
vWF	9.19	2.70	11.54	0.0015
t-PA	–0.23	0.280	0.65	0.42



Legend: RP=Raynaud's Phenomenon; CAOD I=Peripheral Vascular Disease with intermittent Claudication (Fontaine stage II); CAOD III/IV=Peripheral Vascular disease with pain at rest/skin ulcers (Fontaine stage III/IV).

Figure 1. TNF (striped bars) and vWF (open bars) levels before and after correction for age.

be thrombin, but neither the F1+2 prothrombin fragment nor TAT complex, indexes of thrombin generation, nor PAI antigen/activity appeared in our study to be related to TNF levels. It is difficult to understand this latter point, since thrombin generation and PAI production are directly induced by TNF. The difference between physiologic conditions with their relatively low levels of TNF values and the acute response evoked by pharmacologic elevation of TNF in the studies cited might explain why we observed no activation of coagulation. On the other hand, the low sensitivity of the available assay, namely, of thrombin formation, might also explain it. The role of IL-1, which was the same in our group of patients as in healthy controls, deserves further study. As expected, IL-1 and TNF are related, but IL-1 seems not to influence any of the target indexes we investigated.

Conclusions

TNF (and IL-1) is an essential mediator of several immunoinflammatory responses, and its role seems to be important in clinical conditions such as infections or autoimmune diseases. However,

its prothrombotic action also appears to be of importance, as suggested by the present study, in those clinical forms with an atherosclerotic substrate, such as chronic arterial obstructive diseases, and especially during aging.

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patient selection for anticoagulant therapy in coronary heart disease

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Short-term administration of anticoagulants after myocardial infarction is directed toward preventing thromboembolism, is fairly safe and is potentially beneficial in some cases. Long-term administration of anticoagulants in chronic coronary heart disease is directed toward preventing coronary thrombosis, carries an appreciable risk of hemorrhage but may be of significant benefit in selected patients with advanced disease.

■ A quarter century ago, anticoagulant drugs were administered to patients with coronary heart disease (CHD) with high expectations that lives would be saved regularly and in great numbers. At the time, antibiotics were making a great impact against infectious diseases and a similar salutary effect of anticoagulants against thrombotic complications of CHD was anticipated. Initial clinical reports corroborated this optimistic view.[1,2]

Disappointment came gradually over the next decade with clinical experience, a second generation of controlled trials[3-6] and an expansion of knowledge of the complications of CHD. It became apparent that the majority of coronary attacks (angina, arrhythmias and subendocardial infarctions) and complications of CHD (arrhythmias, cardiogenic shock, heart failure and cardiac rupture) are not thrombotic in origin. Thus, most patients with coronary sclerosis die of nonthrombotic causes,[7,8] eg, myocardial infarction without coronary thrombosis; cardiac failure; or one of a host of noncirculatory causes. Anticoagulant drugs therefore may prevent only one complication of CHD, ie, thrombosis.

Selection of patients with CHD for treatment with anticoagulants requires an awareness of the differences between short-term use of these agents after acute myocardial infarction and their long-term use after infarction for coronary sclerosis. The objectives and the degrees of risk of anticoagulant therapy in these two situations are distinctly different (table 1).

Short-Term Use of Anticoagulants After Acute Myocardial Infarction

In studies reported in 1938[7] and 1947,[8] before the advent of coronary care units and the practice of early ambulation, it was determined at autopsy that thromboembolic complications accounted for about one fourth of early deaths following myocardial infarction. The percentage of patients dying from thromboembolism was thought to be about equal to the percentage dying from arrhythmias, cardiac failure, or either cardiac rupture or an extracardiac cause (table 2). The results of the first large trial of anticoagulant therapy in myocardial infarction matched expectations: The hospital mortality among 589 patients receiving anticoagulants was 16% as compared with 23% among 442 control subjects—a 7% reduction.[9] These observations guided the management of patients with acute myocardial

table 1. anticoagulant therapy for coronary heart disease

Form of therapy	Objective	Degree of risk
Short-term after acute myocardial infarction	Prevention of pulmonary and systemic embolization	Fairly low
Long-term for chronic coronary sclerosis	Prevention of coronary thrombosis	Moderately high

infarction for the next decade, ie, through the early 1960s.

From 1961 to 1973, five major trials[3-6,10] of anticoagulant therapy in acute myocardial infarction were reported and, in the aggregate, demonstrated only a 2% reduction in hospital mortality among 1,954 treated patients as compared with 2,028 control subjects—a reduction that may be attributable to chance. However, the incidence of pulmonary and systemic embolic complications recognized during life[4,6,9] and at autopsy[3,9] was reduced significantly—by about a factor of 5—among the treated patients.

It is now believed that the principal causes of death following acute myocardial infarction are arrhythmias and cardiac failure (table 3). With early ambulation, only a small percentage of patients have fatal thromboembolic complications. Thus the potential benefit from anticoagulant therapy is proportionately small. Most clinicians have come to share the view expressed in 1952 by Russek and Zohman[11] that anticoagulant therapy for all patients with acute myocardial infarction is neither necessary nor wise.

It is quite clear that the danger of thromboembolism after an acute myocardial infarction is greatest among poor-risk patients, most of whom can be identified almost immediately. Young patients with pre-infarction angina or uncomplicated subendocardial infarction, for example, as a rule do not benefit from anticoagulant therapy. Here the danger of hemorrhage from anticoagulant therapy probably outweighs the benefit. With each added risk, however, the danger of thromboembolism rises stepwise (figure 1). For elderly poor-risk patients who are extremely ill and bedfast for many days, the chance of a fatal thromboembolism approaches 6%.[7,8] In these patients, anticoagulant prevention of fatal complications by 25% and nonfatal thromboembolism by more than 50% is a very worthwhile objective and a realistic goal. Thus, anticoagulant therapy is recommended for most patients with transmural infarction and for most with subendocardial infarction if additional risk factors are present.

The risk of hemorrhage must be assessed against the potential benefit of anticoagulant therapy. In none of the clinical trials of anticoagulant therapy in acute myocardial infarction reported to date has the mortality been greater among treated patients compared to controls. Fatal hemorrhage has occurred in about one of every 2,000 patients treated.[3-6,9,10] The benefit of anticoagulant therapy outweighs the risk of fatal hemorrhage by a factor of 40 to 1 among all patients with acute myocardial infarction treated with these agents and probably by a higher ratio among those patients in whom thromboembolism is most likely to occur.

Long-Term Use of Anticoagulants for Chronic Coronary Sclerosis

In selecting patients for long-term anticoagulation there has been a great controversy during the last two decades since the British cardiologist Paul Wood recommended anticoagulant treatment indefinitely for all patients with known coronary sclerosis.[12] Many clinicians now select patients for long-term therapy with the same thoughtfulness and care exercised in the choice of patients for coronary bypass surgery. Each patient treated during an acute myocardial infarction should be reevaluated thoroughly at the time of hospital discharge before a decision is reached to continue this medication at home. Long-term anticoagulation poses a greater hazard than short-term therapy, has a different objective and requires full cooperation by the patient. Only a minority of patients with CHD are suitable candidates; many are unreliable, oth rs have contraindications to anticoagulant

table 2. proportions of patients with acute myocardial infarction dying in hospital from various causes: 1950 concept

Arrhythmias	6%
Cardiac failure	6%
Thromboembolism	6%
Cardiac rupture and extracardiac causes	6%

table 3. proportions of patients with acute myocardial infarction dying in hospital from various causes: current concept

Arrhythmias	13%
Cardiac failure	6%
Thromboembolism	2%
Cardiac rupture	2%
Extracardiac causes	2%

therapy and many are too old to be treated safely at home. Finally, large numbers of patients with early CHD are being identified who probably would not benefit from anticoagulant therapy because of the mildness of their disease.

The annual mortality rate after recovery from a single infarction is not remarkably high, equal to approximately eight deaths per 100 patients per year for patients of all ages.[13] Rates are higher for older patients with extensive infarcts and complicating diseases such as hypertension and diabetes. The excess mortality (difference between expected and observed rates) among men between 30 and 70 years of age who survive single infarctions does not exceed five deaths per 100 patients per year according to Beard and associates.[14] They observed an increased mortality with advancing age among patients with single infarctions and a parallel increase in expected deaths due to aging in the general population. Thus a 65-year-old male survivor of a single myocardial infarction faces a 3% risk of death from all causes annually,[15] plus an additional 5% risk associated with clinically evident CHD.

Approximately 50% of survivors of a single myocardial infarction die of a recurrence.[16,17] The remainder die of other cardiovascular diseases and complications, including sudden death without myocardial infarction, plus the entire gamut of noncirculatory diseases. Not all myocardial infarctions are preceded by coronary thrombosis; perhaps two thirds are.[18] Fewer than half the survivors of a single myocardial infarction die of coronary thrombosis, an event which might be averted by effective anticoagulant therapy. Thus, no more than two deaths per year per 100 survivors of a single infarction could be expected to be prevented or postponed by optimal long-term anticoagulant therapy.

These predicted results have been matched by observed results in a collaborative analysis of nine clinical trials involving 1,257 patients treated with anticoagulants and 1,230 matched controls.[19] Among men between 35 and 75 years of age who survived a single myocardial infarction, 11% of those receiving long-term anticoagulant therapy and 13% of the controls died during the first two years of the study.

Considering the economic burden of long-term anticoagulation and the associated risks of hemorrhage, it may be unwise to administer these agents for an extended period to survivors of single myocardial infarctions without evidence of more advanced CHD. However, a very significant reduction in the incidence of re-infarctions among treated patients has been noted.[20-24] During two years, 147 of 949 treated patients and 243 of 939 control patients had re-infarctions of the myocardium. These results make it tempting to administer long-term anticoagulant therapy to totally reliable patients after a single infarction for two or more years with the expectation of a 40% reduction in the risk of re-infarction, considering the dangers of re-infarction for the patient and the associated distress, for both the patient and the family.

The most important information gained from the trials of long-term anticoagulant therapy in CHD is the significant benefit observed among patients with advanced disease,[19] ie, a history of two or more myocardial infarctions or one infarction plus angina pectoris. This benefit is illustrated best by the Veterans Administration Cooperative Study reported in 1969.[24] Among 80 treated patients who had had two or more infarctions, the

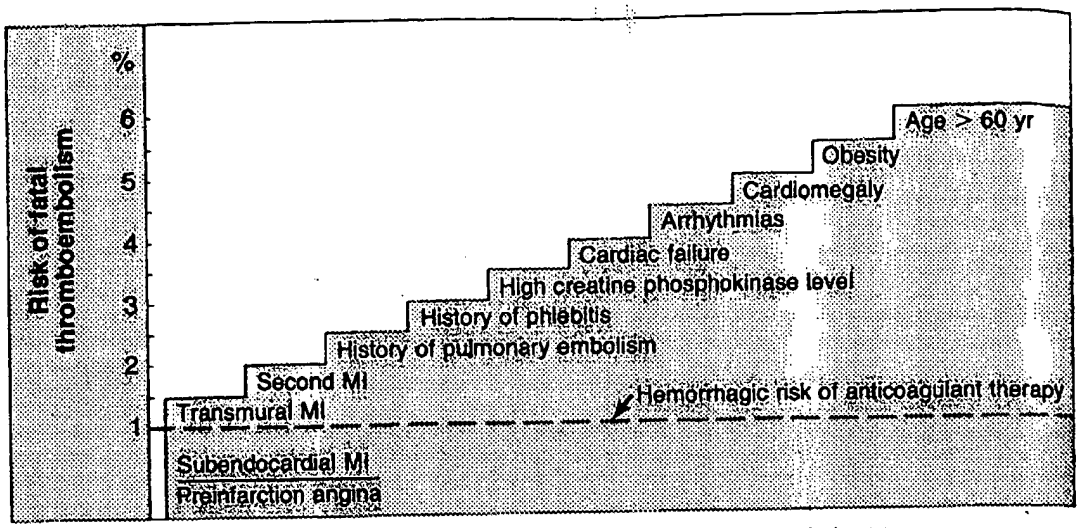

Figure 1. Risk of fatal thromboembolism in relation to hemorrhagic risk of short-term anticoagulant therapy following acute myocardial infarction (MI).[11]

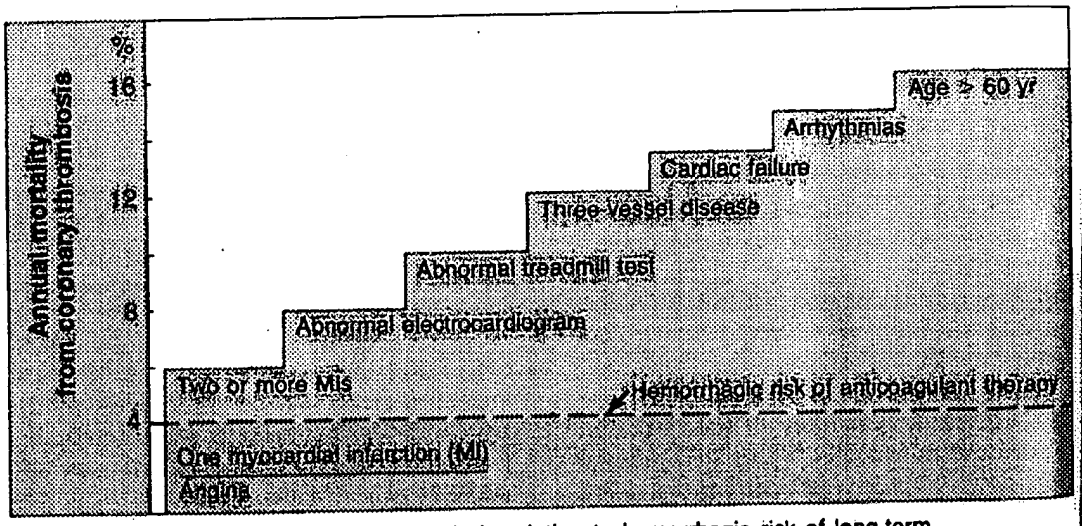

Figure 2. Risk of fatal coronary thrombosis in relation to hemorrhagic risk of long-term anticoagulant therapy for coronary heart disease.[24]

five-year mortality was 38% compared with 54% among 72 controls. The annual mortality in the treated group was reduced by one third and the benefit was sustained beyond five years.

The risk of coronary thrombosis in patients with CHD and the benefit of anticoagulant therapy are directly proportional to the severity of disease (figure 2). The greatest risk of coronary thrombosis obviously exists among patients with far-advanced coronary artery disease, ulcerated plaques and markedly narrowed vessels at multiple sites carrying small volumes of blood at slow flow rates. Severity can be assessed accurately by clinical, electrocardiographic and angiographic studies. Cooperative patients at high risk should be identified. Those who have had two or more myocardial infarctions and those who have had one infarction plus other evidence of advanced CHD should be offered anticoagulant therapy for at least three years following each

coronary event and in most instances, indefinitely. Patients more than 80 years of age, however, should be screened very carefully because of the greater frequency and severity of hemorrhagic complications in this age group.

Summary

Short-term anticoagulant therapy given after an acute myocardial infarction is directed toward preventing thromboembolism and is fairly safe. Long-term anticoagulant therapy prevents coronary thrombosis in selected patients with coronary heart disease (CHD), but carries an appreciable risk of hemorrhage. A decision for or against short-term therapy should be based on an assessment of the immediate risk of thromboembolism. Similarly, the risk of coronary thrombosis should be the major determinant in a decision for or against long-term anticoagulation. The most important information emerging from the clinical trials of long-term anticoagulant therapy in CHD concerns the significant benefit observed among patients with advanced disease. ■

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The Interaction of Activat d Protein C and Thrombin with the Plasminogen Activator Inhibitor R leased from Human Endothelial Cells

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Key words

Plasminogen activator inhibitor – Activated protein C – Thrombin – Fibrinolysis

Summary

The effects of human activated protein C (APC) and thrombin on plasminogen activator inhibitor (PAI-1) released from cultured human umbilical endothelial cells, grown in serum-free ^{35}S-methionine containing medium, were studied in two ways: 1) measurement of PAI-1 activity with an amidolytic assay, and 2) immunoprecipitation of the medium with anti-PAI-1 IgG, anti-protein C IgG or anti-thrombin IgG followed by SDS-PAGE and autoradiography.

Addition of APC or thrombin to the endothelial cell conditioned medium results in a time and concentration dependent loss of PAI-1 activity and in the degradation of PAI-1 from 46 kD into a 42 kD product.

After incubation of the medium with APC in the presence of cells, an additional band of 95 kD was found, which could be immunoprecipitated with both anti-PAI-1 IgG and anti-protein C IgG, indicating the formation of an APC-PAI-1 complex before degradation occurs. No complex could be detected after incubation of the medium with thrombin in the presence of endothelial cells.

Blocking the active sites of APC and thrombin prevented both the formation of APC-PAI-1 complexes and the inactivation and degradation of PAI-1. After removal of the active PAI-1 from the medium, no degradation of the inactive PAI-1 by APC or thrombin could be found.

It is concluded that both APC and thrombin react with the active PAI-1, resulting in inactivation and degradation of PAI-1.

Introduction

The importance of protein C as a physiological regulator of fibrin formation has been established by the finding that inherited deficiency of protein C is associated with an increased risk for the development of thromboembolic diseases (1, 2). In addition to its well documented anticoagulant activity, activated protein C (APC) also exerts profibrinolytic activity, both *in vivo* and *in vitro* (3–8). *In vitro*, APC accelerates whole blood clot lysis (6, 7) and *in vivo*, infusion of bovine APC into dogs (3) or human APC into cats (8) results in increased levels of circulating plasminogen activator. However, in squirrel monkeys no increase in plasma fibrinolytic activity could be demonstrated (4). The precise role of APC in the regulation of fibrinolysis is still not known.

Endothelial cells from human umbilical cord arteries and veins in culture synthesize and release tissue-type plasminogen activator (t-PA) and a component that inhibits both t-PA and urokinase activity (9). This inhibitor is usually present in excess to t-PA (10) and exists in two, immunologically related forms, an active form and an inactive form (which is identical to the so-called "latent" inhibitor) (9, 11). The latter form expresses inhibitory activity after treatment with denaturing agents, such as SDS (12). Both forms have a MW of approximately 46 kD (9, 13). These inhibitors probably have an important regulatory role in controlling the fibrinolytic system of the vessel wall.

We have previously presented evidence that APC and thrombin decrease PAI-1 activity in endothelial cell conditioned medium (14). The molecular mechanism of the interaction between APC and thrombin with the PAI-1 from human endothelial cells is not yet clear.

The availability of anti PAI-1 antibodies (11) allowed us to study further the interaction of APC and thrombin with PAI-1. Because recently, several investigators (14–17) have reported effects of thrombin on the fibrinolytic system, we compared the effects of APC and thrombin on PAI-1 in this study. Using ^{35}S-methionine-labeled endothelial cell conditioned medium we could demonstrate the formation of a complex between APC and PAI-1 and degradation of PAI-1 by thrombin and APC. We also investigated the relative contribution of the active and inactive forms of PAI-1 in the interaction with these proteases.

Materials and Methods

Sterile human serum albumin (HSA) was purchased from the Central Laboratory of the Red Cross Transfusion Service, Amsterdam, The Netherlands. ^{35}S-methionine and En^{3}hance were supplied by New England Nuclear, Boston, MA, USA. Tween 80 was purchased from Merck, Darmstadt, W.-Germany. S-2251, S-2238 and S-2366 were from KabiVitrum AB, Stockholm, Sweden. M199 (Earle's base) supplemented with 20 mmol/l HEPES was obtained from Flow Laboratories Ltd., Irvine, Scotland. Hirudin was purchased from Sigmal Chemical Company, St. Louis, MO, USA.

Proteins

Human protein C (2) and human thrombin (18) were purified as described previously.

Protein C was activated to activated protein C as described by Bertina et al. (2, 18). The final preparation contained less than 1.7 mmol thrombin/mol APC, and no detectable factor X_a (as measured with the specific chromogenic substrate S-2337). No fibrinolytic activity could be detected in the APC preparation, using a t-PA assay with the chromogenic

Abbreviations: t-PA: tissue-type plasminogen activator; PAI-1: plasminogen activator inhibitor released by human endothelial cells in culture (nomenclature according to the recommendations of the ICTH subcommittee on fibrinolysis 1986); ECCM: endothelial cell conditioned medium; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; PBS: phosphate buffered saline (0.14 MNaCl, 0.01 M sodiumphosphate, pH 7.2); RFA: reverse fibrin autography; DIP: diisopropylfluorophosphate.

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substrate S-2251, or with the fibrin plate assay (19). Diisopropylfluorophosphate-inactivated (DIP)-APC was prepared as described previously (14). Residual APC activity, as measured from the amidolytic activity towards S-2366 and the anticoagulant activity in an activated partial thromboplastin time assay, was less than 1%.

Human α-thrombin was prepared and purified as described by Bertina et al. (18). No fibrinolytic activity could be detected in the α-thrombin preparation, using a t-PA assay with the chromogenic substrate S-2251, or with the fibrin plate assay (19).

Hirudin inactivated thrombin was prepared by adding hirudin to the thrombin solution, so that the amidolytic activity of thrombin towards the chromogenic substrate S-2238 was completely quenched.

Human plasminogen was purified from Cohn fraction III by lysine-Sepharose chromatography (20).

Antisera

Anti-protein C IgG was prepared from rabbit antiserum raised against purified human protein C (14). Anti-thrombin IgG was prepared by passing rabbit anti-prothrombin antiserum over a column of DIP-thrombin-Sepharose (18 mg DIP-thrombin/5 gram Sepharose). The anti-thrombin antibodies were eluted from the column with 0.1 M glycine, pH 2.45.

Anti-PAI-1 IgG was kindly provided by Dr. E. D. Sprengers (Gaubius Institute TNO, The Netherlands) (11).

Assays

APC activity was estimated from its amidolytic activity towards S-2366 in a buffer containing 0.03 M Tris, 0.2 NaCl, 0.1 mg/ml ovalbumin and 0.4 mM S-2366, pH 8.5

Thrombin activity was estimated from its amidolytic activity towards S-2238 in a buffer containing 0.1 M Tris, 0.1 M Imidazol, 0.09% HCl, 0.21 M NaCl and 10 mM S-2238, pH 8.3.

PAI-1 activity was quantified by titration of samples with increasing amounts of t-PA, followed by a two-step spectrophotometric assay of the residual t-PA activity as reported by Verheijen et al. (21). The amount of t-PAI-1 activity was deduced from the intersection of the asymptote to the titration curve with the X-axis. One unit of PAI-1 activity is defined as the amount of inhibitor that neutralises one international unit of t-PA activity. One unit PAI-1 equals approximately 2 ng/ml. APC, thrombin or hirudin do not interfere with the PAI-1 assay.

Inactive PAI-1 activity was assessed by SDS-PAGE, followed by reverse fibrin autography as previously described (9, 22). Regions in the SDS-PAGE gel containing inhibitor activity are indicated by the formation of lysis-resistant areas in the indicator gel.

t-PA antigen was determined by the method of Rijken et al. (23).

Endothelial Cells

Endothelial cells from human umbilical cord arteries and veins were isolated by the method of Jaffe et al. (24) and cultured as previously described (25). Cells were grown on fibronectin-coated T25 flasks (Costar, Cambridge, Mass., USA) in M199 supplemented with 10% human serum (not heat-inactivated) and 10% newborn calfserum, 200 μg/ml endothelial growth factor [extracted from bovine hypothalamus as described by Maciag et al. (26)], 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine in a 5% CO_2/95% air atmosphere.

Confluent cultures (0.5 to 0.7 mg cell protein per 25 cm^2 flasks) were washed twice with prewarmed, serum free M199 medium and then incubated for various times (as indicated for each experiment) at 37° C under 5% CO_2/95% air atmosphere in 300 μl or 1000 μl incubation medium, consisting of M199 medium, supplemented with penicillin/streptomycin and glutamine as above plus 1.3 mM $CaCl_2$ (final $CaCl_2$ concentration in the medium was 3 mM), 0.03% human serum albumin and the amount of APC or thrombin indicated for each experiment. After incubation the conditioned media were collected and centrifuged for 5 min at 10,000 g in a Beckman Microfuge centrifuge at 4° C to remove cellular debris. Supernatants were stored at −20° C until further analysis.

When incubations of endothelial cell conditioned medium (ECCM) with APC or thrombin occurred in the absence of endothelial cells, ECCM was prepared as described above, before the proteases were added, and incubations started.

Table 1 Effect of APC or thrombin on the plasminogen activator inhibitor activity present in endothelial cell conditioned medium

		% remaining PAI-1 activity
no protease	t = 0 hours	100
no protease	t = 4 hours	50
APC or DIP-APC	t = 0	100
APC	t = 4 hours	18
DIP-APC	t = 4 hours	50
thrombin or thrombin + hirudin	t = 0	100
thrombin	t = 4 hours	15
thrombin + hirudin	t = 4 hours	48

Endothelial cell conditioned medium, containing both forms of PAI-1 (active and inactive) was incubated for 4 hours at 37° C with 60 nM APC, 60 nM DIP-APC, 50 nM thrombin or 50 nM thrombin + 20 Units/ml hirudin. After the incubation period the PAI-1 activity was assayed according to Verheijen et al. (21). Results are expressed as % of the amount of PAI-1 activity present at t = 0 in the absence of protease.

Radiolabeling of Endothelial Cell Proteins

Incorporation of ^{35}S-methionine into endothelial cell proteins occurred during incubation of endothelial cells with M199 medium (methionine-free) supplemented with HSA, penicillin and streptomycin, APC or thrombin *as above,* and 20–100 μCi ^{35}S-methionine/ml. Radiolabeled ECCM was collected as described above. No morphological changes of the endothelial cells were observed during the incubation period.

SDS-PAGE

SDS-PAGE in slab gels was performed according to the method of Laemmli (27), with 10% acrylamide running gels and 4% acrylamide stacking gels. Molecular weight protein standards for SDS-PAGE (BDH Chemicals Ltd., Poole, England) were used for calibration of the gel system.

For autoradiography, gels were impregnated with an autoradiofluorography enhancer (En^{3}hance), dried and placed on an X-ray film (Kondak AR) for 3–6 days at −20° C.

Immunoprecipitation

The ^{35}S-methionine labeled ECCM was immunoprecipitated with antibodies against PAI-1, protein C or thrombin essentially as described by Kessler (28), with modifications suggested by Bollen et al. (29). Briefly, 150 μl of the labeled medium was preincubated for 20 min at 0° C with 2.5% Staphylococcus aureus (SA). After centrifugation the supernatant was incubated overnight with the appropriate amount of antiserum (10 μg/ml anti-PAI-1 IgG, 15 μg/ml anti-thrombin IgG or 15 μg/ml anti-protein C IgG). The formed complexes were precipitated with SA and after extensive washings with PBS, the pellet was suspended in 50 μl electrophoresis buffer according to Laemmli (27), heated for 3 min at 100° C and centrifuged for 10–20 min in a Beckman Microfuge centrifuge at 10,000 g. The dissolved immunoprecipitate found in the supernatant was used for separation on a SDS polyacrylamide (10%) gel and where indicated, subsequently analysed for inactive PAI-1 by means of reverse fibrin autography (9, 22).

Inactive PA Inhibitor

To inactivate the active PAI-1 (14), the ECCM was incubated at 37° C for 6 hours. After this incubation period, no PAI-1 activity could be detected by using the assay described by Verheijen et al. (21). The ECCM was frozen and stored at −20° C until further use.

Protein Synthesis

Protein synthesis was determined by measuring the ^{35}S-methionine incorporation into the 10% trichloroacetic acid precipitable fraction of radiolabeled ECCM.

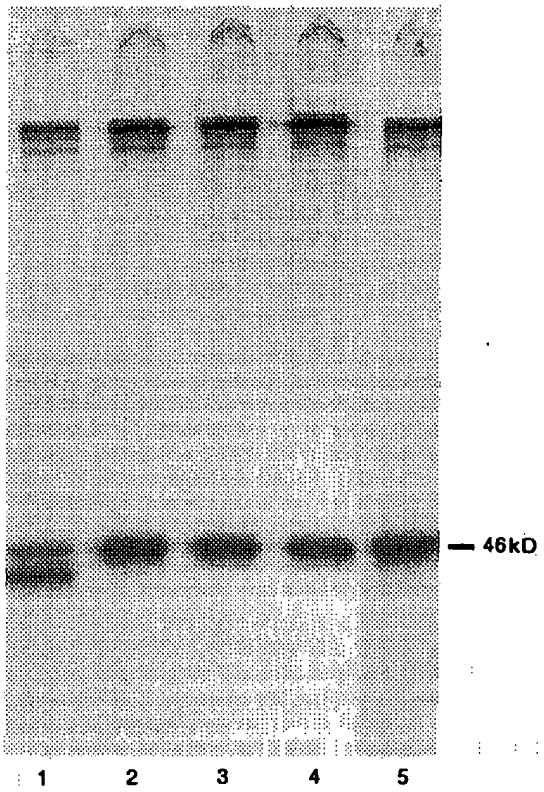


Fig. 1 Effect of APC or thrombin on the inactive PA-inhibitor present in ECCM. A crude preparation of ^{35}S-methionine-labeled inactive PAI-1 was made (lane 5) (see Materials and Methods section) and incubated for 3 hours at 37° C with 60 nM APC (lane 2), 50 nM thrombin (lane 3) or no added protease (lane 4). Lane 1: reference thrombin incubation in the presence of cells (see Fig. 3 for details). After incubation samples were immunoprecipitated with anti-PAI-1 IgG, and run on a SDS polyacrylamide (10%) slab gel. Radiolabeled proteins were visualized by autoradiography

Western Blotting

150 µl conditioned media was first concentrated 3 times with a Centricon™ microconcentrator according to the manufacturers' instructions (Amicon Corporation, Danvers, Mass., USA). The concentrated samples (50 µl) were mixed with Laemmli sample buffer and separated on a SDS polyacrylamide gel according to Laemmli (27). Western blotting with either anti-PAI-1 IgG or anti-protein C IgG was performed according to Towbin et al. (30). For the detection of alkaline phosphatase-conjugated anti-rabbit IgG antibodies on the blots, the method of Blake et al. (31) was used.

Results

Effect of APC and Thrombin on Inactive PA Inhibitor

The effect of APC or thrombin on the inactive form of PAI-1 present in ^{35}S-methionine labeled ECCM was investigated. A preparation of inactive inhibitor (see Materials and Methods section) was incubated with APC, thrombin or buffer for 3 hours at 37° C. The samples were immunoprecipitated with anti-PAI-1 IgG and analysed by SDS polyacrylamide gel electrophoresis. As can be seen in Figure 1, the inactive PAI-1 migrates with a MW of approximately 46 kD. No differences in electrophoresis patterns were observed in the absence or presence of APC or thrombin (lanes 2 and 3). The inactive form of PA inhibitor can be reactivated by SDS after which its presence can be demonstrated by reverse fibrin autography. No qualitative differences in the lysis resistant areas in the indicator gel between the APC, thrombin or buffer incubated samples could be detected by using this technique (data not shown). These results show that APC and thrombin do not react with the inactive PAI-1 present in ECCM.

Effect of APC and Thrombin on Active PA Inhibitor in the Absence of Cells

Incubation of ECCM, containing both forms of PAI-1 (active and inactive form), with APC or thrombin resulted in a reduction of PAI-1 activity (Table 1). This inactivation was active site dependent since DIP-APC or hirudin inactivated thrombin did not show any effect on PAI-1 activity. When the samples were immunoprecipitated with anti-PAI-1 IgG and analysed by SDS polyacrylamide gel electrophoresis, an additional 42 kD band becomes visible in the APC and thrombin incubated ECCM (Fig. 2). No significant amount of radiolabel could be immunoprecipitated with anti-protein C IgG or anti thrombin IgG (Fig. 2), indicating the absence of complexes of PAI-1 with APC or thrombin.

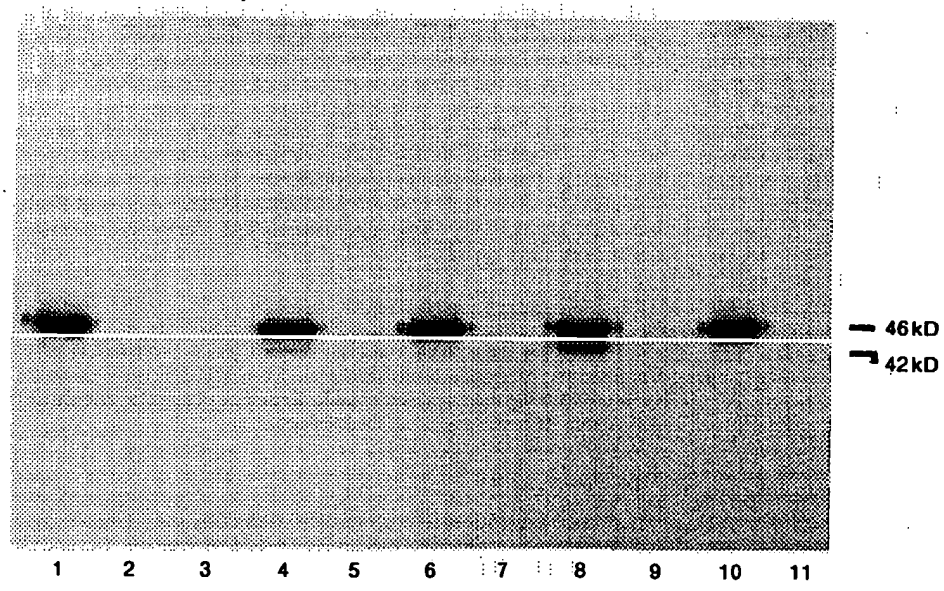


Fig. 2 Effect of APC or thrombin on the PAI-1 present in endothelial cell conditioned medium (ECCM). ECCM (labeled with ^{35}S-methionine) containing PAI-1 activity was incubated for 4 hours at 37° C with 60 nM APC (lanes 4, 5), 60 nM DIP-APC (lanes 6, 7), 50 nM thrombin (lanes 8, 9), 50 nM thrombin + 20 Units/ml hirudin (lanes 10, 11) or an equivalent volume of buffer (lanes 1–3). After 4 hours incubation samples were immunoprecipitated with either anti-PAI-1 IgG (lanes 1, 4, 6, 8, 10), anti-protein C IgG (lanes 2, 5, 7) or anti-thrombin IgG (lanes 3, 9, 11). The immunoprecipitates were separated by SDS-PAGE. Radiolabeled proteins were visualized by autoradiography

Complex Formation Between APC or Thrombin with Active PAI-1

In the experiments described above, no complex between the proteases APC or thrombin with PAI-1 could be detected. This might be due to the low PAI-1 concentration in ECCM, the high K_d of the complexes or an artifactual dissociation of a complex induced by the techniques used.

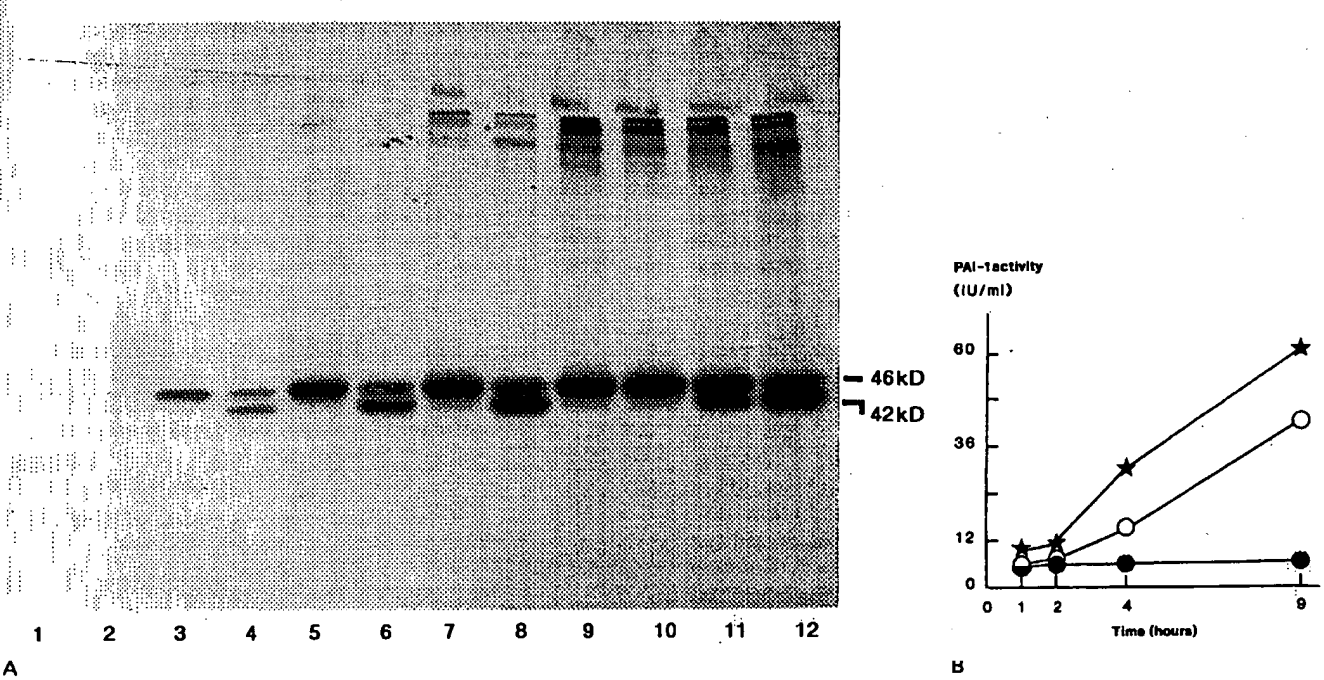
To overcome the problem of inhibitor concentration, endothelial cells were incubated in the presence and absence of APC or thrombin. When we assume that PAI-1 is secreted in an active form and becomes rapidly inactivated (32, 33), we would expect that over a period of time larger amounts of active PAI-1 have been available for interaction with APC or thrombin, when these proteases are added to cultured endothelial cells. After incubation of endothelial cells with APC or thrombin the conditioned medium was immunoprecipitated with anti-PAI-1 IgG and analysed on SDS polyacrylamide gels. The results of a *thrombin* incubation experiment is shown in Figure 3A. The 42 kD product is already visible after a one hour incubation at 37° C (lanes 2 and 4). The longer the incubation period, the more 42 kD product accumulates in the medium. The appearance of the 42 kD product runs parallel with a decrease in PAI-1 activity (Fig. 3B). No radiolabel is detected after immunoprecipitation with anti-thrombin IgG (Fig. 2, lane 9), which suggests that no stable complex between thrombin and PAI-1 was present.

The results of an incubation of endothelial cells with different *APC* concentrations is shown in Figure 4. After immunoprecipitation of the medium with anti-PAI-1 IgG we observe not only the 42 kD product but also a 95 kD product (Fig. 4, lanes 3 + 5). This 95 kD product probably represents a complex between APC and PAI-1 since it can also be immunoprecipitated with anti-protein C antibodies (Fig. 4, lane 6). The 95 kD product could also be detected when a similar sample was analysed on a SDS polyacrylamide gel and subsequently Western immunoblotted with anti-PAI-1 or anti-protein C antibodies (Fig. 5, lanes 3 + 4).

The 42 kD product could also be demonstrated when the medium was immunoprecipitated with anti-protein C IgG (Fig. 4, lane 6). This appears to be due to dissociation of the APC-PAI-1 complex after it has been immunoprecipitated with anti-protein C IgG, because when the immunoprecipitation step was omitted and a similar sample was immunoblotted with the same anti-protein C antibodies, only the 95 kD product (and APC) became visible and no 42 kD product could be detected.

Besides a 42 kD product it appears from Fig. 4 that APC and thrombin incubated cells also show the presence of a low molecular weight product (lanes 3, 5, 6, 7, arrow). This fragment could correspond with the expected 4 kD split product from the 46 kD PAI-1 when it is cleaved by APC or thrombin to a 42 kD and low molecular weight fragment.

The interaction of APC or thrombin with the *active* form of PAI-1 could also be demonstrated indirectly by using a RFA technique after SDS-PAGE of the anti-PAI-1 IgG immunoprecipitated samples. The 46 kD PAI-1 consists of both the active and inactive inhibitor. A small percentage of the latter can be reactivated by SDS and visualised by means of a RFA technique. Regions in the SDS-PAGE gel containing inhibitor activity can be visualised by the formation of lysis resistant areas in the indicator gel. When cells were incubated with APC or thrombin, the lysis resistant area in the indicator gel was decreased as compared to the buffer incubated cells (Fig. 6). Because we previously demonstrated that APC and thrombin have no effect on the SDS-reactivation of the inactive inhibitor, the results of the experiment



Figs. 3A, B Effect of thrombin on plasminogen activator inhibitor in the presence of endothelial cells.
Fig. 3A Immunoprecipitation of plasminogen activator inhibitor. Endothelial cells were incubated for various time intervals with 0, 1, 10 or 50 nM thrombin in the presence of ^{35}S-methionine. After incubation samples were centrifuged to remove cellular debris, immunoprecipitated with anti-PAI-1 IgG, and run on a SDS polyacrylamide (10%) slab gel. Radiolabeled proteins were visualized by autoradiography. Lanes 1, 2: 1 hour of incubation; lanes 3, 4: 2 hours of incubation; lanes 5, 6: 4 hours of incubation; lanes 7, 8: 9 hours of incubation; lanes 9–12: 22 hours of incubation. No protease was added in the samples applied to lanes 1, 3, 5, 7, 9. 1 nM thrombin in lane 10, 10 nM thrombin in lane 11, 50 nM thrombin in lanes 2, 4, 6, 8, 12
Fig. 3B Activity of plasminogen activator inhibitor. After the incubation periods indicated in Fig. 3A the PAI-1 activity in the conditioned media were assayed according to Verheijen et al. (21) (see Materials and Methods section for details). X no protease; 1 nM thrombin gave similar results. O 10 nM thrombin. 50 nM thrombin

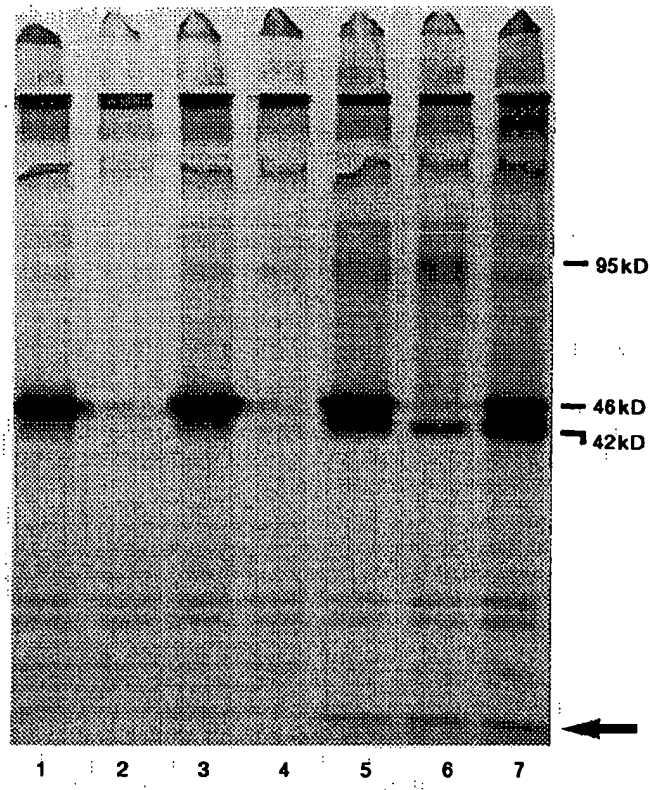


Fig. 4 Effect APC on plasminogen activator inhibitor in the presence of endothelial cells. Endothelial cells, in the presence of ^{35}S-methionine, were incubated for 8 hours at 37° C with 6 nM APC (lanes 3, 4), 60 nM APC (lanes 5, 6) and 50 nM thrombin (lane 7) or no added protease (lanes 1, 2). After incubation, samples were centrifuged to remove cellular debris, and immunoprecipitated with anti-PAI-1 IgG (lanes 1, 3, 5, 7) or anti-protein C IgG (lanes 2, 4, 6) and run on a SDS polyacrylamide (10%) slab gel. Radiolabeled proteins were visualized by autoradiography. The arrow indicates low molecular product

of Figure 6 can be explained by assuming that APC and thrombin react with the active PAI-1 and thus partially prevent the transition of the PAI-1 from the active into the inactive form. The experiment of Figure 6 also shows that the 42 kD degradation product, which was present in the preparations, did not induce a lysis resistant area, which suggests that the 42 kD product cannot be reactivated by SDS.

Discussion

Several investigators have suggested a possible role for APC in fibrinolysis by inactivating a PAI-1 released by endothelial cells (14, 34). These authors have suggested a proteolytic degradation of the plasminogen activator inhibitor by APC or a formation of stable inactive complexes with APC, but no experimental data has been provided to support these hypothesis. Thrombin also is reported to have effects on the fibrinolytic system of endothelial cells in culture (14–17). Addition of thrombin to human endothelial cells in culture results in the induction of t-PA activity (16), whereas Gelehrter et al. (17) reported an increase in PA inhibitor production.

The present study provides experimental evidence for the formation of a SDS stable complex between APC and the active PAI-1 and for the proteolytic degradation of PAI-1 by thrombin.

In the absence of endothelial cells a SDS stable complex between APC and PAI-1 could not be detected, which agrees with our previously reported results (14). This appears to be due to the low concentration of the active form of PAI-1. Indeed, when endothelial cells were incubated with APC, thus allowing a continuous production of active PAI-1 (33), a 95 kD complex between APC and PAI-1 could be demonstrated by immunoprecipitation (Fig. 4) and immunoblotting of the ECCM (Fig. 5). Apparently, upon secretion by the endothelial cells, the active form of PAI-1 may react with APC to form a 95 kD complex. When similar experiments were carried out with thrombin, which also decreases PAI-1 activity in ECCM (14, Fig. 3B), no complex between thrombin and PAI-1 could be detected, neither in the presence nor in the absence of endothelial cells using the immunoprecipitation technique (Fig. 2, 3A). However a 42 kD product appears on the SDS gels, which increases in intensity when longer incubation periods and higher thrombin concentrations are used. This 42 kD product probably represents a degradation product of PAI-1 since it reacts with the same anti-PAI-1 antibodies as PAI-1.

The 42 kD degradation product of PAI-1 also appears in the APC incubated ECCM, both in the presence or absence of cells, but *only* when the medium is immunoprecipitated with anti-PAI-1 IgG or anti-protein C IgG (Figs. 2 and 4) prior to SDS-PAGE electrophoresis. An explanation for detecting a 42 kD PAI-1 degradation product in an anti-protein C IgG formed immunoprecipitate could be the dissociation of the APC-PAI-1 complex after the procedure of immunoprecipitation prior to addition to the gel (see Materials and Methods). This could explain why no 42 kD product is observed when using an immunoblot technique. The much more gentle way in which samples are prepared for the immunoblot (a simple concentration step) might result in less dissociation of the APC-PAI-1 complex prior to addition to the gel. In any case, it is clear that the 95 kD APC-PAI-1 complex could be detected with both techniques, indicating a relatively

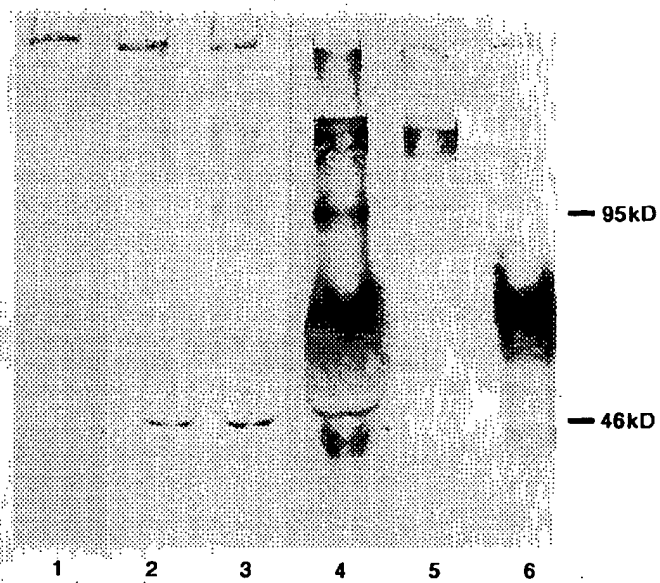


Fig. 5 Detection of complexes between APC and PAI-1 and degradation product of PAI-1 by Western blot. Endothelial cells were incubated for 8 hours at 37° C with buffer (lanes 2, 5), or 60 nM APC (lanes 3, 4). After the incubation period samples were centrifuged to remove cellular debris, concentrated (3×) with a centricon™ microconcentrator (see Materials and Methods section), and blotted according to Towbin et al. (30). Blots were incubated with either anti-protein C IgG (lanes 4, 5, 6) or anti-PAI-1 IgG (lanes 1, 2, 3) for 2½ hours at room temperature. For the detection of the alkaline phosphatase-conjugated anti-rabbit IgG use was made of the technique described by Blake et al. (31). Lanes 1 and 6 reference APC preparation

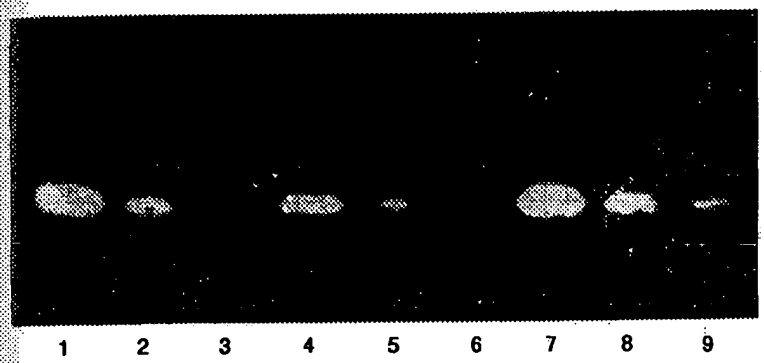


Fig. 6 Effect of APC or thrombin on reverse fibrin autography of plasminogen activator inhibitor. Endothelial cells (in the presence of ^{35}S-methionine) were incubated for 8 hours at 37° C with 60 nM APC (lanes 1, 2, 3), 50 nM thrombin (lanes 4, 5, 6) or buffer (lanes 7, 8, 9). After incubation, samples were centrifuged to remove cellular debris, and immunoprecipitated with anti-PAI-1 IgG. Samples were applied to the gel in Laemmli sample buffer in a non-diluted, 3× diluted and 6× diluted form. The dilutions were performed in Laemmli sample buffer. After SDS-PAGE the gel was further analysed by reverse fibrin autography (see Materials and Methods section). The region in the gel containing inhibitory activity is visible as an opaque, lysis resistant zone in the indicator gel. Lanes 1, 4, 7: undiluted samples; lanes 2, 5, 8: three-fold diluted samples; lanes 3, 6, 9: six-fold diluted samples

stable complex whereas no thrombin-PAI-1 complex could be demonstrated.

In this report we also demonstrated that APC and thrombin react with the *active* form of PAI-1. The formation of APC-PAI-1 complex or the formation of 42 kD PAI-1 degradation product was accompanied by a reduction in the concentration of the inactive PAI-1 as visualized by reversed fibrin autography due to trapping of the active PAI-1 by APC and thrombin (Fig. 5). A preparation of inactive inhibitor alone did not react with APC or thrombin (Fig. 1). It was also shown that the 42 kD product cannot be reactivated upon incubation with SDS (Fig. 5).

The APC-PAI-1 complex formation and the formation of the 42 kD product by thrombin is dependent on the presence of the active sites of APC and thrombin, since inactivated APC and thrombin do not show these effects.

The above presented results are in agreement with the fact that PAI-1 belongs to the serpin family, including mostly serine protease inhibitors (35). Some of these form SDS stable complexes with a protease after which degradation of the inhibitor takes place by the release of a small peptide with MW between 4,000 D and 10,000 D.

The native form of the PAI-1 could be one that exerts inhibitory activity but has not such a stable conformation. After its secretion by endothelial cells it becomes rapidly converted in an inactive form. Both forms have a molecular mass of 46 kD on SDS-PAGE. When APC or thrombin react with the active PAI-1 they form a complex in which the PAI-1 part is degraded to a 42 kD product and a low molecular weight product of approximately 4–10 kD (Fig. 4). Dissociation of the complex into 42 kD-PAI-1 product and the free protease occurs with thrombin, but not as markedly with APC; or schematically:

$$E + I \underset{k_1}{\overset{k_{-1}}{\leftrightarrows}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + I^*$$

in which E represents either APC or thrombin, I the active form of PAI-1 (46 kD), I* degraded PAI-1 (42 kD), EI protease inhibitor complex and EI* protease degraded inhibitor complex.

The k_2 and/or k_3 for the thrombin induced reaction are probably higher than for the APC induced reaction. Recently, Nielsen et al. (36) have shown that urokinase and t-PA are able to catalyse the conversion of PAI from fibrosarcoma HT-1080 cells to an inactive form with an MW slightly lower (~4 kD) than that of the non-converted form and Kooistra et al. (33) have shown, that when t-PA reacts with PAI-1, mainly a t-PA-PAI-1 complex and also a limited amount of 42 kD product is formed. Their results fit in the above model and contribute to the idea that PAI-1 cannot only be degraded by APC, thrombin or t-PA but probably by many more serine proteases.

The sequence of a cDNA coding for the PAI-1 has recently been published by Ny et al. (37) and Pannekoek et al. (38). In an alignment of the amino acid sequence of the reactive centers of PAI-1, α_1-antitrypsin and anti-thrombin III, Ny et al. find an arginine residue as the P1 residue of the PAI-1. This alignment is consistent with the known arginine specificity of APC, thrombin and t-PA. At the moment it would be worthwhile to know the amino terminal sequence of the 4 kD split product of the PAI-1. This will be of great value for determination of the active site of PAI-1.

The physiological significance of the PAI-1 inactivation by APC and thrombin is not yet clear. At least two relatively fast inactivation pathways of PAI-1 exist in vivo: a rapid clearance of the PAI-1 and the reaction with plasminogen activators. The inactivation of PAI-1 by APC and thrombin has been shown to be rather slow and may therefore play a minor role in the in vivo inactivation of PAI-1, unless a mechanism (e.g. specific cofactors) exists which accelerates this reaction at least 100-fold. A cofactor could be protein S which has been shown to be a cofactor for the inactivation of Factor V_a by APC (39), and a cofactor for the acceleration of whole blood clot lysis by APC (7). Recently Lockhart et al. found an acceleration of PAI inhibition by APC when protein S and phospholipids were added. In their experiments they used platelet releasate as a source of PAI (40). Preliminary experiments in our laboratory however show that when protein S (1 unit/ml), phospholipids (4.5 μM) and Ca^{2+}-ions are added to the APC incubation of ECCM in the absence of cells, no acceleration of the PAI-1 inactivation by APC could be detected. These preliminary data seem to be in contrast with those reported by Lockhart et al. (40). However, we have to keep in mind that different sources of PAI were used. Further experiments on the cofactor role of protein S are needed for a better understanding of the physiological role of the interaction of APC with PAI.

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Original Paper

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Plasma Parameters of the Prothrombotic State in Chronic Uremia

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Abstract
We measured plasma parameters of the prothrombotic state, namely thrombin-antithrombin III complex (TAT), fibrinopeptide A (FPA). D-dimer (DD), von Willebrand factor (vWF), tissue-type plasminogen activator (tPA), β-thromboglobulin (βTG), platelet factor 4 (PF4) and serotonin (5HT) in a series of 51 adult patients with chronic uremia: 22 were on maintenance hemodialysis (MHD) and 29 on conservative dietary treatment. Serum tumor necrosis factor α (TNF) was determined as well. Uremics presented significantly higher levels of TAT, FPA, DD, vWF, TNF, βTG and 5HT than normal controls. Patients on conservative treatment showed lower levels of TAT, DD, TNF and βTG than patients on MHD. Our results provide evidence that a prothrombotic state exists in chronic uremia and that MHD patients have a higher degree of hypercoagulation. Both hemodialysis procedure and uremia-related factors are likely to contribute to the hemostatic derangement.

Introduction

The widespread improvement of substitutive and conservative treatment of end-stage renal disease has considerably ameliorated the prognosis of uremic patients. At present, the thrombotic complications, namely ischemic heart attacks, stroke and thromboembolism, have become the predominant causes of mortality in patients with chronic uremia [1]. Hypertension, dislipidemia and secondary hyperparathyroidism are well-known causes of vessel damage, but also hemostatic system abnormalities can play a role. Indeed, high plasma levels of factor VIII procoagulant, von Willebrand factor (vWF) and fibrinogen, low concentrations of antithrombin III and protein C, impaired release of plasminogen activator and reduced survival of circulating platelets have been reported in renal failure [2–14].

On the basis of recent advances in the technique for the measurement of prothrombotic state markers, we have evaluated plasma parameters that directly reflect an activation of the hemostatic system in chronic uremic patients: thrombin-antithrombin III complex (TAT), a marker of

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intravascular thrombin formation; fibrinopeptide A (FPA), a specific indicator of thrombin activity on fibrinogen; D-dimer (DD), a cross-linked fibrin split product; tissue-type plasminogen activator (tPA), the main physiological activator of blood fibrinolysis; β-thromboglobulin (βTG) and platelet factor 4 (PF4), two α-granule-specific proteins released into plasma during platelet stimulation; serotonin (5HT), a vasoactive amine stored in platelet dense bodies and secreted during the release reaction. We have also measured vWF, a protein required for the stable adhesion of platelets to subendothelium and tumor necrosis factor α (TNF), a cytokine derived from monocyte macrophages able to induce endothelial cells to express prothrombotic activities.

So far, most of the investigations concerning hemostatic system alterations in chronic uremia have been carried out in patients undergoing dialysis. In the present study, we have also evaluated patients with end-stage renal disease on conservative treatment.

Patients and Methods

Patients

We studied 51 patients with severe or terminal chronic renal failure. Twenty-nine of them (19 males, 10 females; aged 33–76 years; mean ± SD 54 ± 11), had been on conservative treatment with a very-low-protein (0.3 g/kg/day) low-phosphorus diet supplemented with essential amino acids and ketoanalogues for 6 months at least; serum creatinine ranged from 4.8 to 13.2 mg/dl (mean ± SD 8.2 ± 2.4 mg/dl). Patients with urinary protein excretion higher than 3 g/24 h were excluded.

The other 22 patients (15 males, 7 females; aged 16–78 years; mean ± SD 54 ± 17), were on maintenance hemodialysis (MHD).

During the study, none of the patients received drugs affecting the hemostatic system, with the exception of the heparin administered during the hemodialytic sessions. Subjects with active thrombosis, hemorrhage, fever, infections, diabetes mellitus or liver disease were excluded. Thirty-two members of the hospital staff, matched for age and sex with the patients, served as controls.

Measurement of Hemostatic Parameters

Blood samples were collected by clean venipuncture without stasis using a 20-gauge needle. Samples were discarded if venipuncture was judged imperfect.

Routine blood coagulation tests, namely prothrombin time, activated PTT, thrombin time, fibrinogen assay and platelet count were performed on all patients. In addition, the following hemostatic parameters were measured: FPA: blood samples for FPA analyses were processed as previously described [15]. Plasma FPA was measured by the radioimmunological method according to Nossel [16] using commercial kits. Values were expressed as nanograms/milliliter. TAT; DD; vWF; tPA: immediately after drawing, blood was gently mixed in plastic tubes containing 3.8% sodium citrate solution (1:10, v:v), and within 30 min, the blood samples were spun at 2,000 *g* for 20 min at 4°C.

TAT was determined by an enzyme-linked immunosorbent assay using a specific antibody reacting with a neoantigen expressed by the complex; values were expressed as nanograms/milliliter. DD was measured by an enzyme-linked immunosorbent assay utilizing a monoclonal antibody which recognizes a neoantigenic determinant expressed by the DD but not D fragment; the antibody has an unsignificant cross-reactivity towards native fibrinogen. Values were expressed as nanograms/millimeter. vWF was assayed by an enzyme-linked immunosorbent assay; values were expressed as Units/deciliter. tPA was determined by a commercially available enzyme-linked immunosorbent assay; values were expressed as nanograms/milliliter. In addition, we measured βTG, PF4 and 5HT. Blood samples were processed as previously described [15]. βTG, PF4 and 5HT were determined by radioimmunological methods using commercial kits. Values were expressed as nanograms/milliliters. βTG values were also corrected for platelet count (βTG ratio) according to Zahavi [17].

And, finally, we also measured TNF: TNF was determined in serum using an immunoradiometric procedure. Values were expressed as picograms/milliliter.

Statistical Analysis

All the results were expressed as mean ± SD. Differences between groups were analyzed for significance using Student's t test for unpaired data, differences were considered significant when $p < 0.05$.

Results

The platelet count in uremic patients ranged from 35 to 401×10^9/l with a mean value of $183 \pm 76 \times 10^9$/l, significantly lower than that of normal controls ($p < 0.01$). No relationship was found between platelet count and plasma concentrations of platelet-secreted substances (βTG, PF4, 5HT).

Plasma fibrinogen ranged from 160 to 600 mg/dl with a mean value of 329 ± 100 mg/dl, significantly higher than that of normal controls (227 ± 38 mg/dl).

Other routine coagulation tests (prothrombin time, activated PTT, thrombin time) were within the normal range in all the patients.

Uremics showed significantly raised plasma levels of FPA, TAT, vWF, TNF, DD, βTG and 5HT in comparison with controls (table 1). Most patients exhibited DD values exceeding 240 ng/ml that represents the upper normal limit, as it appears in figure 1. All the patients had βTG values exceeding 59 ng/ml, well above the upper limit of the normal controls (i.e., 40.5 ng/ml). Also the βTG ratio was significantly higher in uremics.

The results were also analyzed as related to the two subsets of uremic patients. Platelet count of MHD patients ($153 \pm 73 \times 10^9$/l) was significantly lower than that of patients on conservative treatment ($206 \pm 79 \times 10^9$/l;

$p < 0.05$). Patients on MHD presented significantly higher concentrations of TAT, DD, TNF and βTG than patients on conservative treatment, as shown in table 2. There was also a trend, although not statistically significant, towards fibrinogen decrease, FPA increase and tPA decrease in the subset of patients on MHD. Also, the subset of uremics conservatively treated had significantly higher levels of fibrinogen, FPA, TAT, DD, vWF, TNF, βTG and 5HT than healthy controls.

Table 1. Plasma levels of the studied parameters in uremics and normal controls (mean±SD)

	All uremics	Controls	p value
FPA, ng/ml	2.64±1.56	1.11±0.47	<0.001
TAT, ng/ml	3.68±1.20	2.12±0.80	<0.001
DD, ng/ml	715±468	120±59	<0.001
vWF, U/dl	140±69	98±19	<0.001
tPA, ng/ml	5.41±3.37	6.68±3.13	ns
TNF, pg/ml	19.1±14.1	7.4±4.8	<0.001
βTG, ng/ml	120±39	25.7±7.4	<0.001
PF4, ng/ml	5.32±2.52	5.32±1.31	ns
5HT, ng/ml	7.33±5.30	3.21±1.57	<0.001

Table 2. Plasma levels of the studied parameters in uremics on hemodialysis and on conservative treatment

	Patients on hemodialysis treatment	Patients on conservative treatment	p value
FPA, ng/ml	3.06±1.59	2.28±1.48	ns
TAT, ng/ml	4.36±1.36	2.94± 0.580	<0.05
DD, ng/ml	851±469	552±415	<0.05
vWF, U/dl	140± 55	139± 77	ns
tPA, ng/ml	4.67±1.87	6.04±4.23	ns
TNF, pg/ml	24.1±17.3	14.1± 7.6	<0.05
βTG, ng/ml	133±46	106±26	<0.05
PF4, ng/ml	5.39±2.34	5.26±2.68	ns
5HT, ng/ml	8.24±4.48	6.30±6.12	ns

Discussion

Reports available to date on hemostatic system abnormalities in end-stage renal disease have a number of limitations.

First, classical laboratory tests of the hemostatic function, well suited to explore the bleeding tendency of chronic uremia, are rather insensitive to detect minor degrees of clotting activation and incapable of evaluating a thrombotic tendency.

Second, to our knowledge, no investigation so far performed concerning the hemostatic function in advanced renal failure has been carried out comparing directly two large series of patients following two different therapeutical strategies (MHD or conservative treatment).

Third, it is not clear to what extent both renal failure on the one hand and hemodialysis procedure on the other hand contribute per se to the hemostatic changes observed in uremics. Indeed, extracorporeal circulation is known to induce a marked alteration in the hemostatic system: namely, during a hemodialytic procedure, fibrin is formed in the dialyzer, circulating activated platelets undergo the release reaction, endothelial cell injury develops and a fibrinolytic response caused by tPA release takes place. These changes have been documented in the course of a single hemodialytic session [10, 13, 18–21], but their long-term effects on the hemostatic equilibrium are not known.

Fourth, plasma levels of circulating substances involved in hemostatic activity, such as low molecular weight proteins and amines, may be influenced by the impaired renal function; on the other hand, some substances are easily removed through the dialytic procedure. Therefore, one must be cautious in the evaluation of hemostatic activities in the presence of decreased renal function.

In our study, we examined two large groups of uremics; one on MHD and the other still on conservative therapy in spite of severe renal failure.

We used newly developed immunochemical tools that can quantitate crucial reactions of the hemostatic system,

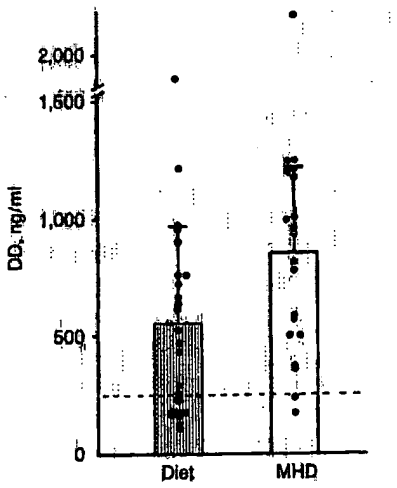

Fig. 1. DD plasma levels of uremic patients on conservative dietary (DIET) therapy and on MHD treatment.

thus allowing a more appropriate characterization of the prothrombotic state.

The conversion of prothrombin into thrombin, the key reaction in the coagulation process, eventually results in the formation of TAT, a high molecular weight product that is metabolized by vascular endothelium. Thus, the plasma levels of TAT do reflect intravascular thrombin generation [22] not affected by renal failure.

The accelerated thrombin formation is also reflected by the increased plasma levels of FPA [16], although the possibility exists that elevated levels of FPA in renal failure are at least partly due to decreased renal catabolism [23, 24].

The enhanced plasma levels of FPA and TAT found in our patients indicate blood hypercoagulability. TAT was more markedly elevated in the subset of MHD patients compared to conservatively treated patients, whereas no significant difference was found between the two subsets in FPA values. The possibility exists that the thrombin generation is underestimated by FPA measurement in MHD patients since a considerable amount of FPA is removed during the hemodialysis session because of the low molecular weight of FPA (1,527 D) [24].

Fibrin polymerization occurs spontaneously as soon as FPA is cleaved from fibrinogen; DD is a stable end product of cross-linked fibrin degradation by plasmin. Therefore, the detection of DD fragment confirms that both coagulation and fibrinolytic pathways have been activated [25], and it is likely that plasma DD levels are hardly affected by renal dysfunction [26]. Thus, the increase in DD plasma concentration, observed in this study, actually indicates enhanced fibrinolysis following coagulation. Accordingly, Nakamura et al. [26] found high values of cross-linked fibrin degradation products and α_2-plasmin inhibitor-plasmin complex in a series of uremic patients on MHD.

The higher DD level we observed in MHD patients, compared to conservatively treated patients, may be due to a more marked activation degree of coagulation and fibrinolysis pathways in MHD. It is tempting to speculate that vascular damage due to renal failure, and exacerbated by a hemodialytic procedure, activates the coagulation system.

Mesangial cells as well as monocytes, producing TNF under appropriate stimulation, could be responsible for the increased TNF concentration we observed in patients with end-stage renal failure. Indeed, monocyte macrophage activation has been postulated in renal failure [27]. Whatever mechanism is operating, recent evidence has been provided indicating that a high concentration of TNF can induce monocytes and endothelial cells to express prothrombotic activities, namely tissue factor, the main trigger of blood coagulation, simultaneously depressing antithrombotic activities such as heparan sulfate and thrombomodulin. Thus, raised serum levels of TNF can contribute to blood hypercoagulation.

Our results confirm the marked increase in vWF antigen in uremics [3, 4]. Increased plasma levels of vWF antigen are regarded as reflecting a release reaction by vascular endothelial cells to certain stimuli or even vascular injury [28]. Whatever mechanism is implicated for vWF increase, high plasma concentrations of vWF can enhance platelet adhesion to vascular subendothelium, a crucial step in the pathogenesis of atherosclerosis [29, 30].

In spite of laboratory signs of hypercoagulation, tPA was not increased in our series of uremics, suggesting an inadequate tPA release in response to fibrin formation [8, 9, 31].

Mean platelet count in our patients was significantly lower than in healthy controls, suggesting platelet overconsumption with inadequate production, as documented by others [12].

As concerns platelet-released materials, an increased plasma concentration of βTG and 5HT has been observed in our patients; conversely, PF4 was in the normal range in the same samples.

It is well known that βTG and PF4, two platelet-specific α-granule proteins, are released into plasma in similar amounts during the process of platelet activation [32, 33]; however, PF4, once secreted, is rapidly taken up by endothelial cells which are endowed with specific binding sites [34], whereas circulating βTG is predominantly cleared by renal parenchyma as a tubular protein [35]. The elevated βTG values found in our patients could be due to an increased release of βTG into the plasma as a consequence of accelerated platelet consumption into the bloodstream or, alternatively, to the failure of the kidneys to clear βTG produced by the natural attrition of senescent circulating platelets or a combination of both.

High βTG with normal PF4 values have been previously observed by other authors in uremic patients on MHD [19, 23, 35]. Hemodialytic treatment has been shown to result in a further increase in βTG antigen in the plasma, suggesting a stimulation of platelets exposed to cuprophane membranes [19]. In this study, patients on MHD presented higher βTG levels compared to the patients on conservative treatment; hence, chronic hemodialytic treatment per se appears to exacerbate βTG elevation. Accordingly, the lower platelet count we found in MHD patients, compared to patients on conservative treatment, is consistent with an accelerated platelet consumption. The enhanced plasma concentrations of βTG might play a role in atherogenesis, as βTG seems to be provided with chemotactic and mitogenic activity [35].

Only few data concerning the metabolism of 5HT in chronic uremia are currently available. As circulating 5HT appears to be predominantly catabolized in the renal parenchyma [36], impaired degradation in the renal parenchyma may account for a raised plasma level of 5HT in our series of uremics. The raised plasma levels of 5HT could provide conditions for exaggerated serotoninergic amplifications, thereby facilitating platelet thrombus formation, atherosclerosis development and arterial hypertension maintenance [37].

Taken as a whole, our results provide evidence that a prothrombotic state does exist in chronic uremia. The hemostatic system alterations we observed in chronic uremia might play a role in the pathogenesis of atherothrombotic complications. However, long-lasting prospective investigations are required to assess this point.

Both dialysis procedure and uremia appear to contribute to the hemostatic derangement, since even patients in dietary treatment show abnormalities of the hemostatic system suggesting a prothrombotic state.

Since some hemostatic parameters indicating blood clotting activation, namely TAT, DD, βTG and TNF are altered to a lesser extent in the uremic patients on conservative treatment compared to uremic patients on MHD, the question arises if regular hemodialysis can accelerate the development of thrombotic complications: this is a matter of great concern, because thrombotic accidents are the leading cause of death in uremics at present [1]. According to our results, conservative treatment could exert an important protective role on thrombotic tendency, because it can postpone the onset of hemodialysis therapy and/or can reduce the frequency of hemodialysis sessions [38–40].

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Pathophysiology of coronary thrombosis

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Detailed knowledge of the pathophysiology as well as the dynamic nature of coronary thrombus formation provides a valuable tool for correct management and proper adjunctive therapy in patients with acute coronary syndromes. Coronary thrombosis is in the majority of cases caused by disruption or fissuring of an atherosclerotic plaque. At the lesion thrombogenic material will be exposed to the flowing blood leading to activation of platelets and the formation of a platelet clot. Simultaneously, the coagulation system is activated resulting in increased thrombin formation. Thrombin is a key mediator in arterial thrombosis, due to its effect on both platelets and fibrin generation. Thrombin contributes to the stabilization of an initially loose platelet clot by generating cross-bound fibrin within the thrombus. During the course of an acute coronary syndrome, the patient presents changing chest pain and dynamic ischaemic ECG findings. This is likely to be related to the dynamic nature of the pathophysiology. The presence of a non-occlusive coronary thrombus may deprive the myocardium its normal blood flow and oxygen supply, leading to ischaemic pain. During lysis or embolization, blood supply may be restored, but the presence of thrombus fragments in the microcirculation holds the potential to sustained interference with myocardial metabolism. The emboli contain activated platelets which release vasoconstrictors that may compromise the microcirculation. Recurrent thrombus formation at the lesion site may result in occlusion of the artery adding to the dynamic nature of the clinical presentation. In conclusion, platelets, the coagulation system, and the endothelium cause a dynamic process of intermittent occlusion, vasospasm and embolization of thrombus material.

Key words: acute myocardial infarction, atherosclerosis, coagulation, coronary heart disease, platelets, thrombosis, embolization

Introduction

Coronary artherosclerosis develops slowly and is, in general, a benign disease. However, its clinical feature may suddenly change and initiate severe life-threatening acute myocardial ischaemia. Clinically, this gives rise to the acute coronary ischaemic syndromes, unstable angina pectoris, acute myocardial infarction and sudden coronary death. Studies based on autopsy [1–5], angiography [6], and angioscopy [7–9] have shown that the formation of a coronary thrombus superimposed on an atherosclerotic plaque, leading to total or subtotal occlusion of the artery, and in some cases peripheral embolization [3], is the key event that causes the change in symptoms and prognosis.

The initial event in coronary thrombus formation is usually disruption or fissuring of the plaque [1–5]. At the site of plaque rupture, platelets adhere to the artery wall and release vasoconstrictory and aggregatory substances. A platelet-thrombus is formed, the coagulation system is activated, and the end-product is a stable coronary thrombus consisting of aggregated platelets and fibrin (Fig. 1). Plaque type (composition), rather than plaque size (stenosis severity), is the most important determinant of the development of the thrombus mediated acute coronary syndromes; lipid-rich and soft plaques are more dangerous than collagen-rich and hard plaques because they are vulnerable to rupture and highly thrombogenic after disruption. Detailed knowledge on the pathophysiology of coronary thrombus formation may provide tools for prevention and treatment of the ischaemic syndromes. Clearly, for interventional cardiologists performing percutaneous coronary interventions where plaque rupture, subsequent thrombus formation and embolization into the microcirculation often occurs, such knowledge is of great importance.

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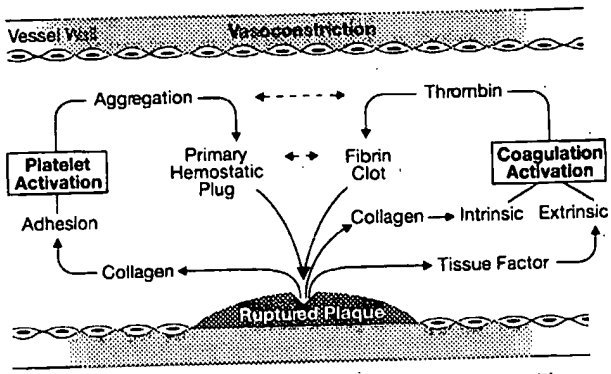

Figure 1. Thrombus formation following plaque rupture. Plaque fissuring causes exposure of thrombogenic material to the flowing blood which will lead to platelet activation and the formation of a platelet clot. The coagulation system is activated simultaneously, mainly by tissue factor, resulting in thrombin formation. Thrombin is a key mediator in thrombosis as it is both a strong platelet activator as well as a precurser of fibrin. Interwoven fibrin fibrils will stabilize the loose platelet clot.

This paper will briefly summarize and update this field, but will also focus on the role of platelets and coagulation in order to form a basis for the understanding of proper initial anti-thrombotic therapy in patients with coronary thrombosis.

The coronary artery

The endothelium

The endothelium is a metabolic cell layer assumed to protect the vessel from adhesion of platelets and leucocytes. The endothelium produces several vasoactive substances such as the vasodilators, prostacyclin and nitric oxide and vasoconstrictors like thromboxane A_2, angiotensin 2, and endothelin-1. Prostacyclin and nitric oxide also have platelet inhibitory effects and together with factors stimulating fibrinolysis like tissue plasminogen activator, these substances play a role in protection of the vessel wall against thrombosis.

Endothelial cell injury, in lesion prone areas, appears to be the initial event in atherosclerosis, leading to accumulation of oxidized LDL and the formation of fatty streaks. The endothelial dysfunction is also associated with increased cell adhesion, mainly monocytes transmigrating into the plaque. Monocytes derived macrophages start endocytosis of oxidized LDL and thereby give rise to the lipid filled foam cell—a hallmark of the mature atherosclerotic plaque.

The vulnerable plaque

As the name 'atherosclerosis' implies, mature plaques consist typically of two main components: soft, lipid-rich atheromatous 'gruel' and hard, collagen-rich sclerotic tissue. The sclerotic component is in general the most voluminous constituting more than 70% of an average stenotic coronary plaque [10]. A thick fibrous cap of collagen and other extracellular components stabilizes and protects the plaque against disruption. In contrast, the atheromatous component is by far the most dangerous because it destabilizes the plaque, and makes it vulnerable and susceptible to rupture and thrombosis [11]. In accordance, a critical atheromatous component is usually found in culprit lesions from patients with acute coronary syndromes [12].

Patients with ischaemic heart disease usually have many atherosclerotic plaques in their coronary arteries. There is no simple relation between the size of a plaque (or stenosis severity) and its vulnerability to rupture [13]. Little is known about what regulates the development of a mature plaque into a vulnerable or a stable plaque and whether these processes are reversible.

Inflammation is likely to play an important role in the initiation and progression of atherosclerosis and thrombosis. High plasma fibrinogen levels as well as increased levels of other inflammatory mediators such as C reactive protein, amyloid A protein and interleukin 6 have also been associated with coronary heart disease and a high risk of acute coronary events [14, 15]. Data from the Physicians Health Study indicate that base-line plasma concentration of C-reactive protein can predict the risk of myocardial infarction and stroke not only in patients with ischaemic heart disease, but also in apparently healthy men [16]. Moreover, in the same population the relative risk of future myocardial infarction among those with increased levels of *both* CRP *and* total cholesterol were greater than the product of the individual risk associated with increased CRP or total cholesterol level [17]. In men, with angiographically verified coronary heart disase, CRP, CD-8 expression on lymphocytes, ICAM-1, and antibodies to oxidised-LDL were found to be determinants of endothelium-dependent vascular dysfunction [18]. These observations suggest that endothelial dysfunction may be related to chronic inflammation or infection.

Culprit lesions responsible for acute coronary syndromes contain significantly more macrophages than lesions responsible for stable angina pectoris (14% versus 3% of plaque area occupied by macrophages) [19]. Using immunohistochemical techniques van der Waal *et al.* demonstrated that macrophages and adjacent T-lymphocytes were activated, indicating ongoing disease activity [20]. The same group has shown an increase in the percentage of IL-2R positive T-lymphocytes in culprit lesions from patients with acute coronary syndromes, suggesting that acute activation or amplification of the immune response in the plaque may trigger the onset of acute coronary events [21].

The composition, vulnerability, and thrombogenicity of individual plaques vary greatly without any obvious relation to risk factors for clinical disease, except

perhaps serum cholesterol. Elevated ratios of total cholesterol to high density lipoprotein cholesterol —was found in male patients who died of plaque rupture. Hypertension, smoking status, age, glycosylated haemoglobin values, and race were not associated with the presence of vulnerable or ruptured plaques, but smoking seemed to predispose patients to acute coronary thrombosis regardless of the underlying plaque morphology [22]. In women, age >50 years, cholesterol was found to be independently associated with the occurrence of vulnerable plaques and smoking was associated with plaque erosion [23].

The presence of several components appear to be associated with plaque disruption: (a) a large atheromatous lipid core, (b) a thin fibrous cap with underlying inflammatory and immune responsive cells (macrophages), and (c) a relatively reduced number of smooth muscle cells and consequently reduced collagen content [11]. Autopsy studies have shown that a lipid core occupying 32% or more of the plaque volume are seen in plaques that are disrupted compared to 5–12% in non-disrupted plaques [24]. Similarly, the majority of disrupted plaques undergoing thrombosis have a lipid core occupying more than 40% of the cross-sectional area of the plaque [25].

Macrophages are capable of degrading extracellular matrix by phagocytosis or by secreting proteolytic enzymes such as plasminogen activators and matrix metalloproteinases, which may digest the matrix component of the fibrous cap, leading to thinning and predisposing it to disruption [26, 27]. The mechanical strength of the plaque cap is a vital component of plaque stability and it depends mainly on thickness, collagen content, and the amount of other tissue proteins. A decline in smooth muscle cell density will inevitably lead to a decline in collagen synthesis and thinning of the cap, making it more susceptible to rupture [28].

Coronary plaques are constantly stressed by a variety of mechanical and haemodynamic forces that may precipitate or 'trigger' disruption of vulnerable plaques. Physical exertion and emotional stress could, for example, trigger plaque disruption via surges in sympathetic activity with an increase in blood pressure, pulse pressure, blood flow (shear forces), heart rate, and coronary tone [29].

Plaque disruption

Plaque disruption is a common event which often occurs during the development of atherosclerotic lesions and it is in the majority of cases clinically silent [11]. Following disruption variable amounts of luminal thrombosis and/or haemorrhage into the soft gruel occurs, causing rapid growth of the lesion. Autopsy data indicate that 9% of apparently healthy persons have disrupted plaques (without superimposed thrombosis) in their coronary arteries [30]. The numbers increase to 22% in persons with diabetes or hypertension [30]. One or more disrupted plaques, with or without superimposed thrombosis, are usually present in coronary arteries of patients dying of ischaemic heart disease [1, 2].

The risk of plaque disruption depends on both intrinsic properties of individual plaques (their vulnerability) and extrinsic forces acting on plaques (rupture triggers). The former predispose plaques to rupture, while the latter may precipitate disruption if vulnerable plaques are present.

The coronary thrombus

Platelet reactivity and inhibition

Platelets are small anucleate, discoid, highly specialized blood cells. The membrane phospholipids contain the substrate for the synthesis of the proaggregatory prostaglandin thromboxane A_2. Intraplatelet membrane systems, the surface connected canalicular system, the dense tubular system and the circumferential band of microtubules, play important roles in the regulation of platelet size, contraction and granule release. The dense granules contain the proaggregatory substances, serotonin and calcium ions. The α-granules contain platelet derived growth factor (PDGF), platelet factor 4, β-tromboglobulin, fibrinogen and plasminogen activator inhibitor.

When endothelial damage occurs either spontaneously or as a result of percutaneous coronary intervention, exposure of the subendothelial thrombogenic milieu to circulating blood allows deposition of platelets and formation of an initial hemostatic plug. Adherent platelets can then be activated by a variety of physiological stimuli (e.g. thrombin, collagen, adenosine diphosphate (ADP), high shear stress), leading to the expression of a full repertoire of prothrombotic platelet activities, most notable among which is the conversion of inactive platelet receptor glycoprotein IIb-IIIa to a ligand-receptive conformation. The final common pathway to platelet aggregation and vascular thrombosis involves cross-linking of activated glycoprotein IIb-IIIa receptors on adjacent platelets by fibrinogen (Fig. 2).

Throughout the last four decades numerous attemps to measure the reactivity of circulating platelets have been published. These platelet function tests include measurement of the cutaneous bleeding time, platelets aggregometry in platelet-rich plasma or in whole blood, platelet volume measurement, measurement of thromboxane and prostacyclin metabolites, measurement of platelet release proteins (platelet factor 4 or β thromboglobulin), platelet integrin expression by flowcytometry and others. Recently, a rapid bedside device for assessment of platelet glycoprotein IIb/IIIa inhibition has been developed [31].

There is some evidence that a systemic activation of coagulation and increase in platelet reactivity occurs in the acute phase of myocardial infarction and in unstable angina pectoris [32]. Patients with acute myocardial

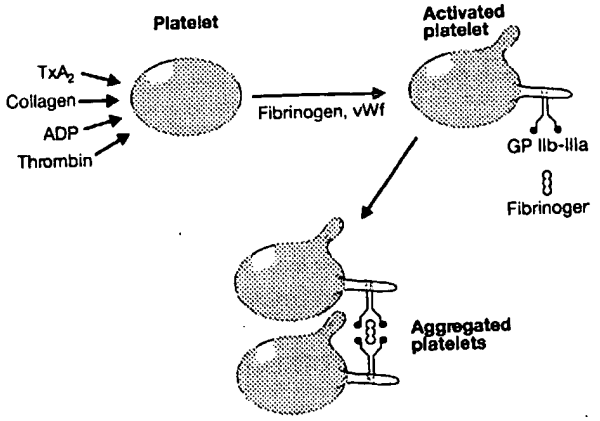

Figure 2. The glycoprotein (GP) IIb/IIIa receptor and platelet aggregation. Activated platelets present GP IIb/IIIa receptors on the surface membrane. The receptor binds circulating fibrinogen and crosslinks the adjacent platelets as the final pathway to platelet aggregation.
TxA_2: thromboxane A_2; ADP: adenosine diphosphate; vWf: von Willebrand factor.

infarction have a shorter bleeding time [33, 34] and a higher mean platelet volume than controls admitted with chest pain without signs of infarction [35]. Since large platelets are more active haemostatically than small ones, this points to an increased reactivity of the circulating platelets at the time of the acute event [32]. Also, the synthesis of the pro-aggregatory thromboxane A_2 is increased in the acute phase of acute myocardial infarction and in unstable angina pectoris [36]. A high mean platelet volume [37] and increased spontaneous platelet aggregation [38] have been shown to be risk factors for re-infarction and death in patients surviving myocardial infarction.

Although, the evidence based on results measured by these tests, points to an increased reactivity of circulating platelets in patients with acute myocardial infarction and unstable angina [32] and also in patients undergoing coronary interventions [39] the concept of a prothrombotic state in patients with acute coronary syndroms may be challenged. First of all, any *in vitro* assessment has limitations, e.g. the platelet sample measured may not be representative for the platelets circulating in the coronary artery and also the environment (presence of anticoagulants, different concentration of, e.g. calcium ions) may be different. Some of the tests have a low accuracy and precision, whereas others are very tedious and difficult to perform, and, therefore, none of these tests have so far been used for individual risk assessment in patients, nor for routine guiding of choice and dose of antiplatelet therapy. Therefore, in our opinion the strongest evidence that platelets are of importance for the formation of coronary thrombus following spontaneous or interventional plaque rupture, is the fact that aspirin (which-inhibits thromboxane A_2 formation) ticlopidine and/or clopidogrel (inhibits the platelet ADP-receptor) and the intraveneous glycoprotein IIb/IIIa antagonists abciximab, eptifibatide and tirofiban, are effective in reducing cardiac events/mortality in patients with unstable angina pectoris and acute myocardial infarction and in patients undergoing percutaneous coronary intervention.

Coagulation

The acute coronary syndromes are characterized by constantly varying symptoms and findings (chest pain and dynamic ischaemic ECG changes), which are likely to correspond to the underlying pathologic processes [40]. Rupture of an atherosclerotic plaque will lead to blood exposure of thrombogenic substances, including tissue factor, von Willebrand Factor (vWF), and collagen from the lipid rich core. vWF and collagen directly stimulate the formation of a platelet clot. Tissue factor will also cause platelet activation, but this is caused by ignition of the coagulation system leading to the formation of thrombin, which is one of the most powerful platelet agonists.

Platelets, when activated, secrete ADP and TxA_2 which will reinforce platelet aggregation, and they will express increased amounts of phospholipids on their surfaces, which will promote coagulation by supporting anionic assembly of the coagulation factor complexes [41].

Although there is no doubt that platelet activation is a key event in arterial thrombosis, the importance of an activated coagulation system has been demonstrated by improved outcome in patients with acute coronary syndromes following thrombin inhibition by heparin/low molecular weight heparin (LMWH) and oral anticoagulants [42, 43].

Coagulation is triggered when activated factor VII (VIIa) binds to tissue factor and this complex activates factors IX and X. In the presence of calcium, factors IXa and Xa bind to their cofactors on the surface of activated platelets (factor VIIIa and Va respectively) to form the intrinsic tenase and prothombinase. Activation of factor X and conversion of prothrombin to thrombin by prothrombinase results in a burst of thrombin generation [44]. Thrombin initiates the conversion of a soluble monomeric protein, fibrinogen to the insoluble protein fibrin. The newly formed fibrin monomers spontaneously polymerize into long chains creating a stable three dimensional structure: an insoluble fibrin clot (Fig. 3).

The dynamic aspects of symptoms and clinical findings in acute coronary syndromes may reflect the dynamics of a growing thrombus. During coronary occlusion the patient is likely to present chest pain and/or ECG changes. However, due to lysis/embolization the coronary artery will become reperfused resulting in pain relieve and improvement of ECG changes.

The thrombus is extremely thrombogenic on its own, as it contains a reservoir of active thrombin leading to activation and aggregation of platelets as well as re-activation of the coagulation system [45]. The surface

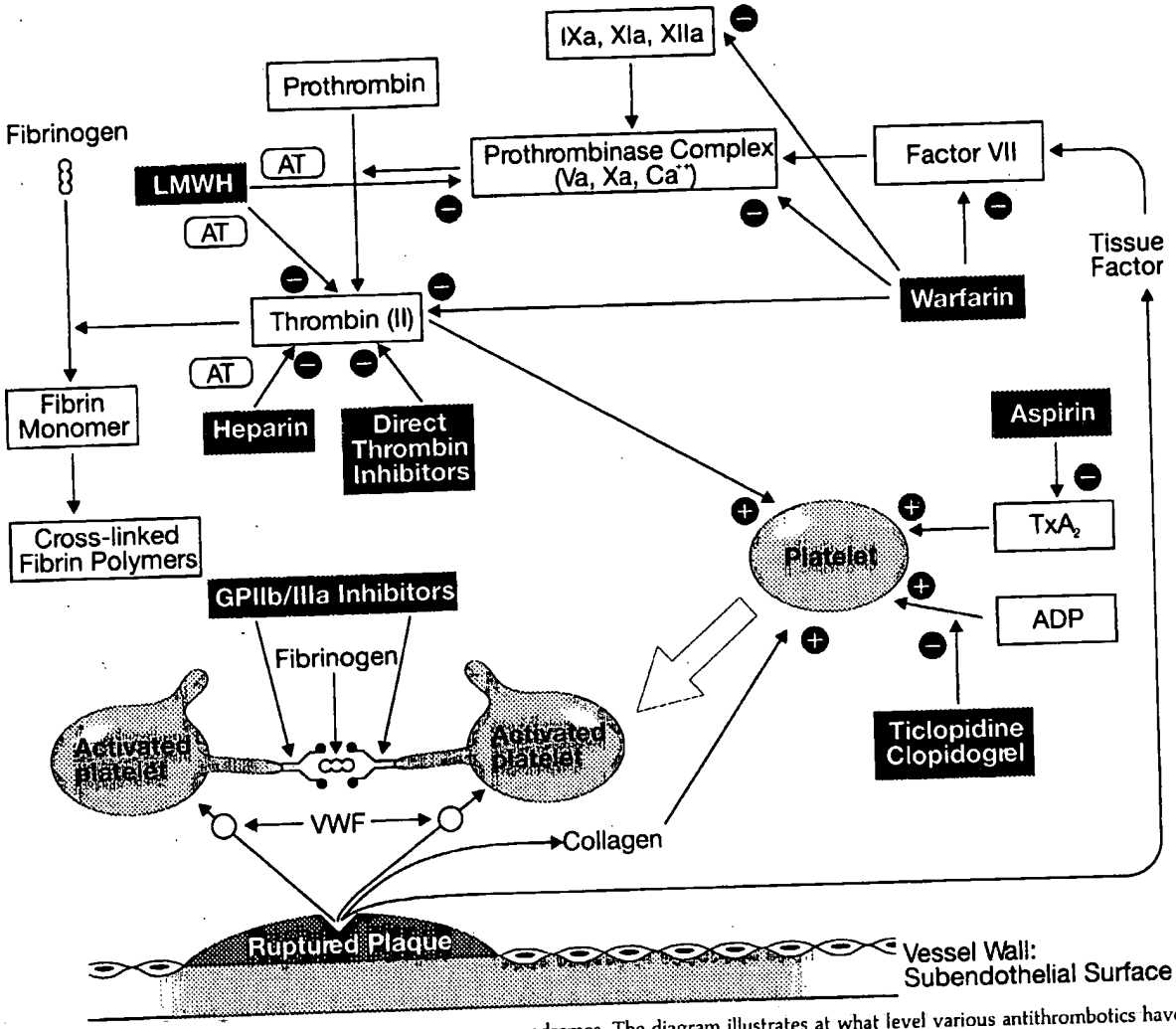

Figure 3. Pharmacological interventions in acute coronary syndromes. The diagram illustrates at what level various antithrombotics have an effect on thrombus formation and what can be obtained by combining two or more anti-coagulant/platelets therapies.
vWf: von Willebrand factor; GP IIb/IIIa: Glycoprotein IIb/IIIa; AT: antithrombin; LMWH: low-molecular-weight heparin; TxA_2: thromboxane A_2; ADP: adenosine diphosphate.

of the thrombus is covered by a layer of activated platelets with a large number of glycoprotein IIb/IIIa receptors on the outer membrane [46] All of these mediators may cause additional thrombus formation resulting in re-occlusion of the artery.

Emboli from the thrombus containing activated platelets stimulate thrombus formation and vasoconstriction in the microcirculation. Furthermore, fibrin-bound thrombin can be found in these aggregates, capable of activating factor XIII, rendering the platelet aggregates more resistent to lysis [47].

In conclusion, the interplay between platelets, the coagulation system and the endothelium cause a dynamic process of intermittent occlusion, vasospasm, and embolization of thrombus material (Fig. 1).

Coronary spasm and peripheral embolization

In patients with acute myocardial infarction, coronary angiography has revealed that occlusion may be intermittent and relieved by infusion of nitrates, indicating that spasm of the coronary artery may add to the occlusion caused by coronary thrombus [48].

Autopsy studies indicate that embolization of the coronary thrombus or of atherosclerotic debris from the ruptured plaque [3, 4] may occur. Although this phenomenon was described already in the early 80s it has not gained proper attention until now. During the last 20 years the focus have mainly been on plaque rupture and coronary thrombosis in the epicardial arteries resulting in detailed information of these pathophysiological mechanisms.

Interestingly, new clinical data strongly suggest that peripheral embolization occurs frequently in patients with acute myocardial infarction [49]. Several lines of clinical evidence supports that peripheral embolization may be important:

(1) In patients undergoing percutaneous intervention, the presence of periprocedural infarcts diagnosed as

release of myocardial enzymes may be related to embolization to the microcirculation and a poor outcome [49].

(2) A significant reduction in the incidence of these periprocedural infarctions can be obtained by treatment with intraveneous glucoprotein IIb/IIIa blockers.

(3) In patients with ST-elevation infarcts the absence of ST-resolution or decreased myocardial blush rate after primary PTCA are associated with a poor outcome despite the presence of TIMI-III flow in the infarct related artery [50, 51].

(4) Adjunctive therapy with abciximab in ST-elevation infarct patients receiving thrombolytic therapy (reduced dose t-PA) [52] or undergoing PTCA [53] causes resolution of ST-elevation in more patients and also improves myocardial perfusion evaluated by the myocardial blush rate.

These exciting new data are, at present, initiating several studies addressing the importance of the perfusion of the myocardium at the microcirculatory level in order to improve our knowledge and basis for adjunctive pharmacological or interventional treatment in patients with acute myocardial infarction.

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